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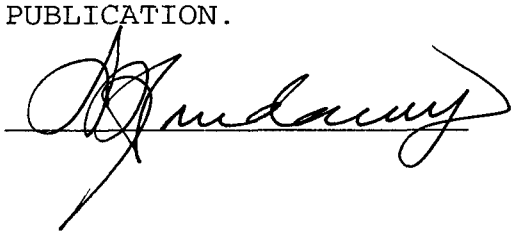

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13. ABSTRACT ***(Maximum 200 Words)***

Splice variants of the homing receptor CD44 may mediate metastasis formation by various tumors through interaction with the cytokine osteopontin, which mediates cell attachment or migration. We have identified the mechanism of cell motility as a two step process involving both major osteopontin receptors. We have further characterized this process by studying signal transduction associated with ligation of each receptor. Because osteopontin structure can be extensively modified on the posttranslational and posttranscriptional levels with substantial consequences for its function we are now analyzing the osteopontin transcripts from various cancer cells for alterations.

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INTRODUCTION

Malignancies display invasive behavior that is conveyed by homing receptors (and associated signal transduction molecules), their ligands (mostly cytokines), and secreted extracellular-matrix degrading proteases. The topology of metastasis formation is determined by the specific identity of the homing receptors expressed on the tumor cell surface and their ligands (Weber/Ashkar 2000). Although the mechanisms regulating such invasive tumor cell migration and subsequent implantation are incompletely understood, there is abundant evidence that the cytokine osteopontin can mediate the dissemination of tumor cells. It constitutes the major phosphoprotein secreted by transformed cells (Senger et al. 1980; Senger et al. 1983; Senger et al. 1989; Chambers et al. 1992) and its levels correlate with metastatic potential (Craig et al. 1990; Senger et al. 1989). Transfection of tumor cell lines with osteopontin increases their malignant phenotype (Denhardt/Guo 1993) while transfection with osteopontin antisense oligonucleotides yields populations with reduced malignant potential (Behrend et al. 1994; Gardner et al. 1994). Induction of osteopontin has been shown to be sufficient for metastasis formation by mammary epithelial cells (Barraclough et al. 1998; El-Tanani et al. 2001).

Splice variants of the homing receptor CD44 mediate metastasis formation by various tumors. Expression of the cytokine osteopontin has been associated with the malignant potential of tumor cells. We have identified a novel receptor/ligand interaction between CD44 and osteopontin that mediates cell attachment or migration. Both molecules are subject to multiple posttranscriptional and pottranslational modifications and our data suggest that these alterations modulate binding. Organ-preference in metastasis formation may be based on specific recognition via homing receptors and modifications in CD44/osteopontin interaction may provide a potential molecular explanation. To assess why breast cancer metastasizes predominantly into bone, we are investigating modifications in both proteins that allow interaction.

BODY

Specific Aim 1: Identification of the posttranslational modifications in both proteins which are permissive for interaction. Tasks2/3. biological functions of metastasis associated gene products are extensively regulated at the posttranscriptional and posttranslational levels (Weber/Ashkar 2000). Consistently, osteopontin from various cellular sources may have diverse structural characteristics (Kon et al. 2000). The main physiologic source of osteopontin is $CD4^+$ T-cells. Macrophages may also produce osteopontin after stimulation with lipopolysaccharide but with distinguishable biological consequences. The macrophage-generated osteopontin is structurally different from the T-cell derived molecule, possibly due to loss of part of its sequence by alternative splicing (S. Ashkar and G.F. Weber, unpublished observations). Studies in osteopontin$^{-/-}$ gene targeted mice (Crawford et al. 1998) have suggested the existence of structural and functional differences between tumor-derived osteopontin and the osteopontin forms that are relevant for host defenses. Evidence suggests that tumor-derived osteopontin is unique (i.e. structurally different from osteopontin derived from non-transformed cells) and lacks important domains (Figure 1). As a case in point, an osteosarcoma secreted a smaller form of osteopontin than the predominant product secreted by non-transformed bone cells (Kasugai et al. 1991). Malignant cells often secrete hypophosphorylated osteopontin variants (Shanmugam et al. 1997) or a splice variant that has a deletion in its N-terminal portion (Kiefer et al. 1989) and this molecule may contribute to metastatic spread (Weber et al. 1997) by inducing cell migration. Concomitantly, hypophosphorylated or alternatively spliced osteopontin could ligate CD44 on macrophages leading to chemotaxis (Weber et al. 1996) and suppression of IL-10, but would possibly engage its integrin receptors less efficiently, so that substantial amounts of IL-12 would not be secreted (Ashkar et al. 2000). Due to the inability of tumor-derived osteopontin to associate with the extracellular matrix (Rittling et al. 2001) its cytokine functions (dependent on its presentation in soluble form (Adler et al. 2001)) may be particularly

prominent. Thus, expression of structurally altered, spliced or hypophosphorylated osteopontin by cancer cells may, among other functions, represent a mechanism of immune evasion.

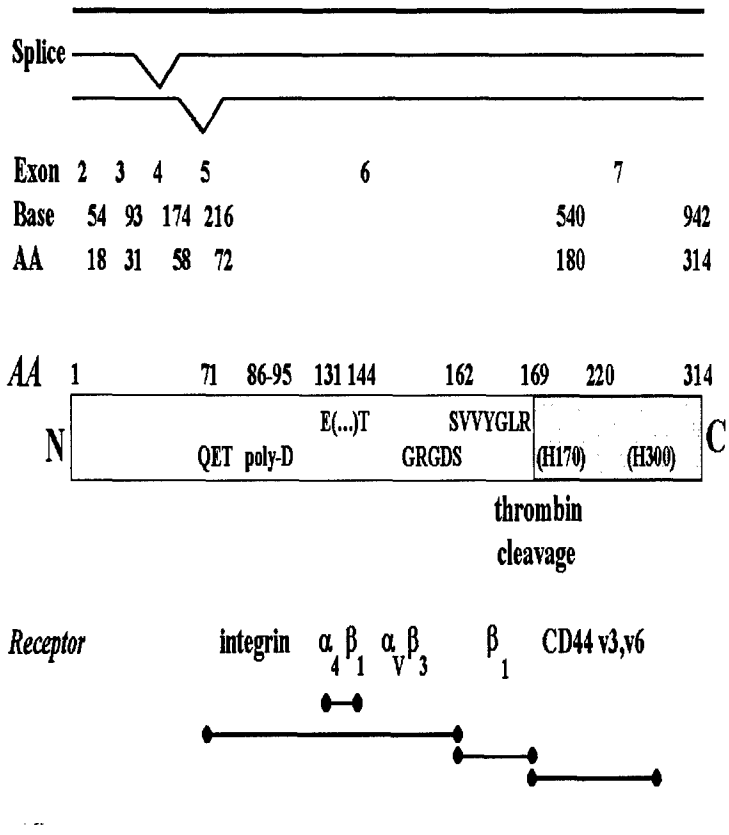

Figure 1: Structural characteristics of the osteopontin gene product. *Top: The gene has 6 translated exons. Sequences for spice variants of exons 4 and 5 are deposited in Genbank. Middle: The protein contains two main domains, a N-terminal fragment contains the integrin binding sites, while the CD44 binding site lies on the C-terminal domain. Bottom: The integrin binding site covers the sequence GRGDS. The smallest integrin $\alpha_V\beta_3$ binding peptide identified by us starts at AA71. Binding to β_1-containing integrins occurs through the non-canonical sequence SVVYGLR, unless the β_1 chain is paired with α_4, in which case the binding site ranges from AA131 to AA144. We have found the CD44v6 binding site to cover the region from AA169 to AA220. Heparin-bridges between osteopontin and CD44v3 may be formed via the heparin binding sites on AA170 and 300. The scheme is not drawn to scale.*

Several reports (Kon et al. 2000; Kasugai et al. 1991; Shanmugam et al. 1997; Kiefer et al. 1989; Weber et al. 1997), some of them recent (Crawford et al. 1998), have suggested that there are structural and functional differences between tumor secreted osteopontin and host osteopontin. Host osteopontin may protect from tumors while osteopontin produced by cancer cells confers metastatic potential (Crawford et al. 1998; Denhardt/Chambers 1994; Feng et al. 1995). We had reason to hypothesize that the structural variations in osteopontin from various sources might originate not only in posttranslational modifications, but also on the level of RNA editing. We have therefore analyzed the osteopontin messages in various normal and transformed breast epithelial cell lines by RT-PCR, cloning, and sequence determination (Figure 2). In transformed epithelial and mesenchymal cells (MDA-MD-

435, 21PT, 21NT breast cancers, LnCAP prostate cancer, SAOS-2 osteosarcoma). In these experiments, cells are starved (serum-free medium), treated with PMA (a potent inducer of osteopontin expression in many cells) or cultured in complete (serum-containing) medium. Osteopontin RNA is extracted, amplified by RT-PCR, cloned and sequenced. We have found the expression of two forms in malignant, but not benign cells. Interestingly, normal breast epithelial cells (generously provided by Dr. Vimla Band; Liu et al. 1996; Ratsch et al. 2001) express low or moderate amounts of standard osteopontin, but no smaller transcript. After immortalization with the human papillomavirus oncogene E6, however, expression of two osteopontin bands is observed. The two bands were identified as the wild-type osteopontin and a splice variant missing the third translated exon (exon 4, because exon 1 is untranslated (Behrend et al. 1993)). The identified transcripts correspond to the sequences of "osteopontin-a" and "osteopontin-b", which have been deposited in GenBank (Accession numbers D28759 and D28760) but have not been thoroughly studied. Published reports on the expression or function of splice variants are limited to the observation of three types of osteopontin RNA messages in human gliomas (Saitoh et al. 1995) and to the existence of a splice variant that has a deletion in its N-terminal portion (Kiefer et al. 1989). It may be important to note that it is not unexpected for tumors to express multiple variant transcripts of metastasis-associated genes. Similar observations have been made for the homing receptor CD44. Tumors may express up to nine distinct spliced forms of this gene (Matsumura/Tarin 1992). Only certain CD44 splice variants support metastasis (Gunthert et al. 1991), while the standard form acts as an inhibitor of dissemination (Tanabe et al. 1995). It can be hypothesized that similar relationships may apply for standard osteopontin and its splice variant. This might account for the apparently conflicting observations that osteopontin produced by cancer cells (the unique form being osteopontin-b) supports their dissemination, while host osteopontin (osteopontin-a) may contribute to anti-cancer immune surveillance.

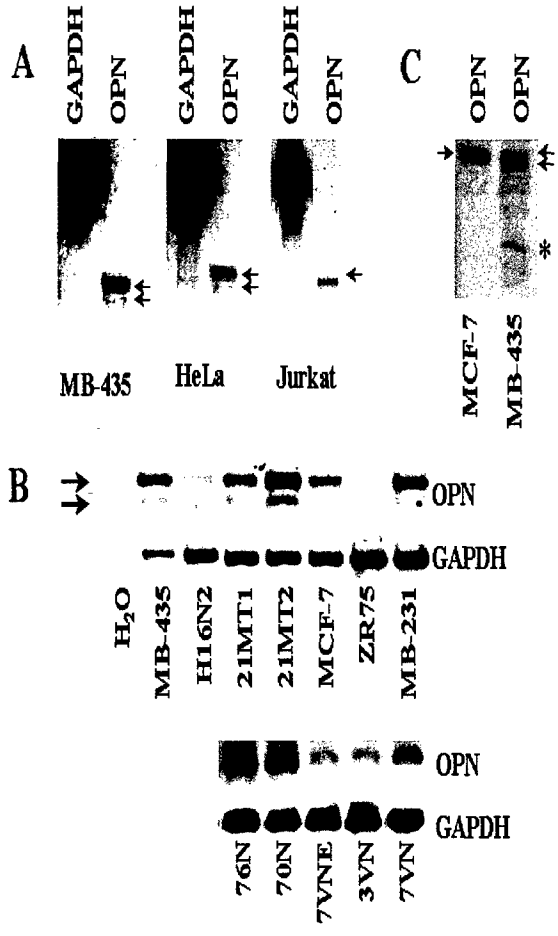

Figure 2: Expression of osteopontin splice variants in multiple tumor cell lines. *A,B) RNA was extracted from various cell lines, was reverse transcribed, and used as template in PCR reactions. Primers for osteopontin (OPN) amplified a 616 bp segment from the 5' end of the transcript. Alongside every other cell line, MDA-MB-435 cDNA was amplified to mark the two osteopontin bands (not shown). Resulting double bands (MDA-MB-435 cells, HeLa cells, 21MT cells, not shown: Saos-2 cells) were cloned and sequenced. No template (not shown) and GAPDH served as controls. A) In malignant breast cancer (MDA-MB-435) and lymphoma (HeLa), two osteopontin bands are amplified. In T-cells (Jurkat) one band is obtained. B) (top panel) Osteopontin expression in breast tumor cell lines. Two bands are seen in the malignant cells (MDA-MB-435, 21MT1, 21MT2, MDA-MB-231). In benign cells (H16N2, MCF-7, ZR-75) one or no band is obtained. (bottom panel) Normal breast epithelial cells express low or moderate amounts of standard osteopontin (76N, 70N, 7VNE, 3VN, 7VN). In contrast, breast epithelial cells immortalized with the HPV oncogene E6 (81E6, M2E6E7, 16E6P) express two transcripts of osteopontin (not shown). C) The number of transcripts detected by RT-PCR corresponds to the number of protein bands on Western blots from cell lysates. The * indicates a likely cleavage product that is very commonly observed on Western blots for osteopontin.*

Because osteopontin is not spliced in benign tumor cells or in T-lymphocytes the domain encoded by exon 4 may be essential for host defense. The splicing deletes a polypeptide that does not have any known functional motifs and interferes only minimally with the previously defined binding region for integrin $\alpha_V\beta_3$ (Figure 2). A homology search in the protein database (pdb) has yielded only limited homology with other polypeptides, whose origins range from viral to xenopus-derived. No specific function can be inferred from the homology search (Table 1).

aatgctgtgtcctctgaagaaaccaatgactttaaacaagag		Osteopontin
ttacgacacaggagacttctttggttactgaaatttgttctc		exon 5
N A V S S E E T N D F K Q E		
N A I K A R N T C D F T Q E	BAA19654.1	polyprotein (virus)
A V S S E L T D Q Y K K E	BAA89487.1	nHAg Na/H antiporter (b. subtilis)
V N S E K T N D L K	BAB07214.1	dihydrodipicholinate red.(b. sub.)
N S V S V E E E A D F K	AAF42364.1	pep-protein transf. (n. mening.)
A V A S E E T D D	AAA85501.1	recombination protein (s. pyog.)
N A I S V N E T N N F	AAB88973.1	RNA polymerase (a. fulgidus)
L S S E E T P D F K	AAC07698.1	phosphoglycerate dehyd. (a. aeol.)
N K V E E G E S N D F K D D	CAA89528.1	DNA polymerase (s. cervisiae)
A V L S E E E N D F	AAB23989.1	Hir1p (s. cervisiae)
N L T S S D E T N S F S K	CAB90776.1	DNA polymerase (s. pombe)
A V L S E E E N D F	P32479	Hir1 (yeast)
M S S Q G T N E F K N E	CAB79136.1	receptor kinase like (a. thaliana)
V Q S E Q T N D M K R	AAF54538.1	CG6471 gene prod. (d. melanogaster)
N D S K S E E Q D D M K Q E	AAF53703.1	CG10568 gene prod. (d. melanog.)
A S A T R E K N D F K S E	CAA30104.1	acetyl choline rec.γ (xenopus)

Table 1: Sequence comparison of osteopontin exon 4. *A standard protein Blast search without filter for lo complexity and with high expectation value identifies polypeptides with 50-60% identity (underlined). Th functional characteristics of the retrieved sequences are diverse. Motifs are not unambiguously identified.*

We have found an osteopontin splice variant to be expressed in breast cancer cells, but not in benign breast tumor cell lines. Even though no unique structural characteristics have been identified for the spliced exon, an existing literature has described structural (migration on SDS-PAGE) and functional (metastasis versus host defense) differences between tumor-derived and host-derived osteopontin that may be explained by differential splicing. A recent publication (Rittling et al. 2001) has reported on the inability of tumor secreted osteopontin to mediate cell adhesion to the extracellular matrix, providing a possible criterion for differentiating standard and spliced osteopontin.

We will follow up on these preliminary observations. If confirmed, we will analyze how the defined sequence changes affect osteopontin binding to CD44. We are also setting up experiments to

immunoprecipitate osteopontin from breast cancer cells (MDA-MB-435 and MDA-MB-231, which secrete osteopontin constitutively) and from bone-derived cells (the target for breast cancer cell invasion) cultured in serum-free medium. We will attempt to analyze the partially purified protein by mass spectrometry for phosphorylation and glycosylation. The Dana-Farber Cancer Institute has a molecular biology core facility that performs similar analyses routinely and has performed studies of posttranslational modifications for us previously. It should be noted, however, that the complexity of the starting material often allows only partial characterization of the protein.

Specific Aim 2: Investigation into the roles of CD44 and osteopontin in metastasis formation.

Task 5. We have back-crossed CD44 knockout mice with mice bearing mutant p53 or APC tumor suppressor genes to assess the influence of CD44 on tumorigenicity. Mice with point mutations in tumor suppressor genes, $APC^{+/min}$ bred on C57Bl/6 background or $trp53^{+/tm1}$ on C57Bl/6 background, were obtained from Jackson Laboratory. Either $APC^{+/min}$ mice or $trp53^{+/tm1}$ mice were mated with $CD44^{-/-}$ mice that had been back-crossed from 129 to C57BL/6 for 4 generations (Su et al. 1992). The genotypes were assessed using PCR from genomic DNA (Schmits et al. 1997; Jacks et al. 1994; Su et al. 1992) and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody IM7 (Pharmingen). Siblings were housed in groups of 1-4 per cage at the Redstone Animal Facility (DFCI) in alternate 12-hr light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered ad libitum and room temperature was kept at 25°C. The colony was frequently tested for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee and the mice were frequently seen by a veterinarian.

Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the CD44 gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One $trp53^{+/tm1}CD44^{-/-}$ mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from $trp53^{+/tm1}$ mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 $CD44^{+/+}$ mice and 1 metastasis in 4 $CD44^{-/-}$ mice. One $CD44^{+/+}$ mouse also displayed a macroscopically visible metastasis in the spleen. All 6 $CD44^{+/+}$ mice had multiple osteosarcoma metastases while in 4 $CD44^{-/-}$ mice only one individual lung metastasis was detected. Consistently, CD44 expression was prominent in the osteosarcomas of $CD44^{+/+}$ mice.

The absence of CD44 does not alter the phenotype of benign tumors. The intestinal polyps caused by mutation in the APC gene grow non-invasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia. Histology was performed on the largest intestinal polyps from each $APC^{+/min}$ mouse to assess malignancy. Consistent with previous reports, these tumors are non-invasive as judged by intact basement membranes in all cases. No metastases were observed in other organs (3 histologic sections per organ). These results were not affected by the presence or absence of CD44. Histologic findings included ectopic hepatic hematopoiesis and bone marrow siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps.

In all tumors studied, the absence of CD44 gene products did not affect tumor incidence or survival. Therefore, CD44 gene products are not essential for tumor incidence and growth, but are important in regulating metastasis formation. The manuscript has been accepted for publication by Cancer Research (see Appendix).

Task 7. One reportable outcome from last year's report was the characterization of osteopontin mediated homing as a two-step process mediated by the two main receptors, CD44 and integrin $\alpha_V\beta_3$ (Weber et al. 2001). In an effort to better understand the mechanisms by which osteopontin mediates cell invasion and survival through its receptors CD44 and integrin $\alpha_V\beta_3$, we have investigated signal transduction events induced by the cytokine after ligation of each receptor. Two manuscripts are in preparation. (see Appendix)

Additional information: At the time of last year's annual report, two manuscripts ("Stress response genes: the genes that make cancer metastasize" and "Molecular mechanisms of tumor dissemination in primary and metastatic brain cancers") were in press. A copy of each original reprint is enclosed with this report.

KEY RESEARCH ACCOMPLISHMENTS

* Identification of CD44 as a metastasis gene that does not affect tumor incidence or growth

* Characterization of signal transduction events associated with ligation of CD44 and integrin $\alpha_V\beta_3$ by osteopontin

* Observation of an osteopontin splice variant selectively expressed in malignant tumors.

REPORTABLE OUTCOMES

Weber GF, Bronson R, Ilagan J, Cantor H, Schmitz R, Mak TW. 2001. Absence of CD44 prevents sarcoma metastasis but causes susceptibility to Hodgkin-like lymphomas. Cancer Research, in press.

Weber GF, Ashkar S. 2001. Ligation of CD44 by osteopontin activates the phosphatase SHP-1. Manuscript in preparation.

Weber GF, Ashkar S. 2001. Two distinct signal transduction pathways associated with ligation of integrin $\alpha_V\beta_3$ by osteopontin. Manuscript in preparation.

CONCLUSIONS

1. CD44 gene products mediate metastasis formation of malignant tumors, but do not contribute substantially to tumor incidence or growth

2. The cytokine osteopontin mediates migration and invasion of tumor cells through a division of labor between its two main receptors, CD44 and integrin $\alpha_V\beta_3$.

3. The osteopontin effect on tumor cells is associated with unique signal transduction events that are only now beginning to become evident.

4. Splice variants of osteopontin are selectively expressed in malignant tumor cells, but not in benign tumor cells.

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Ligation of CD44 by Osteopontin Activates the Phosphatase SHP-1

Key terms: Osteopontin, macrophages, breast cancer, chemotaxis, phosphatase, phosphatidylinositol 3-kinase

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ABSTRACT

The cytokine osteopontin acts predominantly on macrophages, its dysregulation may contribute to the malignant phenotype of various cancers. Osteopontin has two domains for engagement of CD44 or integrin receptors respectively which it uses to coordinately mediate migration and activation. Signal transduction through CD44 leads to chemotaxis and inhibition of IL-10 production. It involves a G-protein and the phosphatase SHP-1 but is independent of protein kinase A or phosphatidylinositol 3-kinase. SHP-1 causes the dephosphorylation of PI 3-kinase. PI 3-kinase is important in cell adhesion and spreading and it is incativated through dephosphorylation by SHP-1. This induces de-adhesion and may be a prerequisite for chemotaxis. This signal transduction pathway is active in macrophages and in cancer cells.

INTRODUCTION

The homing receptor CD44 is a highly complex molecule that may express up to 10 variant exons in the extracellular domain which modulate the affinity to various ligands. Due to alternative splicing, a CD44 variant with extended cytoplasmic tail can be generated which may be the predominant form (Stamenkovich et al. 1989). Ligation of this molecule regulates cytoskeletal changes and cell motility. Various CD44 variants bind to distinct ligands resulting in diverse biological responses. Engagement of CD44 by the major characterized ligand hyaluronate leads to cell aggregation and possibly inhibition of apoptosis (Yu et al. 1997). Engagement of CD44 expressed on macrophages by osteopontin mediates chemotaxis and inhibition of IL-10 secretion (Weber et al. 1996; Ashkar et al. 2000). The associated signal transduction process is largely unknown. Two dominant mechanisms for CD44 associated signal transduction may be phosphorylation and remodeling of the cytoskeleton. Either or both processes could be activated after ligation by osteopontin.

CD44 from human fibroblasts and hematopoetic cells is phosphorylated on serine, but not on tyrosine or threonine. Trypsin digestion of the protein yields at least seven phosphopeptides (Isacke et al. 1986; Carter and Wayner 1988). The cytoplasmic tail of CD44 contains 3 potential serine phosphorylation sites, Ser316, Ser323, and Ser325 (Camp et al. 1991; Neame et al. 1992), however serine 316 is is close to the cell membrane and likely not a major site of phosphorylation. Serine phosphorylation and a very small extent of threonine phosphorylation of murine T-cell lymphoma derived CD44 can be mediated by protein kinase C, but not by cAMP-dependent or -independent kinases, and leads to

enhanced ankyrin binding (Kalomiris and Bourguignon 1989). Binding of soluble or plated hyaluronate has been shown to be independent of CD44 phosphorylation (Uff et al. 1995).

Phosphorylation in CD44-dependent signal transduction may also be mediated by receptor associated tyrosine kinases. The Src-family non-receptor protein tyrosine kinases such as Lck, Fyn, Lyn and Hck were shown to be coupled to CD44 via sphingolipid-rich microdomains (lipid rafts) of the plasma membrane (Ilangumaran et al. 1999; Rozsnyay 1999; Skubitz et al. 1998; Ilangumaran et al. 1998). In T-cells, antibody ligation of CD44 induces association with $p56^{lck}$ and consecutive tyrosine phosphorylation of ZAP-70 (Taher et al. 1996). Hyaluronate may engage a signal transduction cascade emanating from CD44 to Ras, PKC zeta, and I-κB kinase 1 and 2 with consecutive activation of NF-κB (Fitzgerald et al. 2000).

CD44 in human peripheral blood monocytes associates with the cytoskeleton which is enhanced by cross-linking but reduced by PMA stimulation (Geppert et al. 1991). This may reflect indirect influence of PMA on CD44. It has been proposed that CD44 may be associated with the cytoskeleton via a linker protein that associates with the transmembrane or extracellular domains of CD44 and may regulate CD44 interaction with the cytoskeleton in a manner that is sensitive to stimulation by phorbol esters (Neame et al. 1992). ERMM proteins bind to CD44 (Tsukita et al. 1994; Legg/Isacke 1998; Yonemura et al. 1998; Zohar et al. 2000) and form a connection between this receptor and the cytoskeleton.

It has been controversial whether phosphorylation and association with the cytoskeleton are related and it is unknown how each corresponds to engagement of CD44 by various ligands. Association of CD44 with the cytoskeleton involves direct or indirect association with actin and may correlate with the degree of CD44 phosphorylation. In resident macrophages, NP-40 soluble CD44 is phosphorylated whereas cytoskeletally associated CD44 is not; elicited macrophages contain only NP-40 soluble phosphorylated CD44. Alkaline phosphatase can remove phosphate from CD44 in resident and elicited macrophages alike and the phosphorylation sites as judged by phosphopeptide- and phosphoaminoacid analysis appear to be similar in resident and elicited macrophages (Camp et al. 1991). Phosphorylation of CD44 requires serines 323 and 325 and is abolished in a tailless mutant but it does not regulate membrane localization or cytoskeletal interaction in human epithelial cells since tailless mutants, but not mutants of the serine residues 323 or 325, display changed surface distribution compared to the wildtype (Neame et al. 1992).

Here we describe a coordinated process of phosphorylation and dephosphorylation to be associated with engagement of CD44 by its physiologic ligand osteopontin. These mechanisms contribute to cytoskeletal rearrangement and enhancement of motility.

MATERIALS AND METHODS

Cell lines. MH-S is a macrophage cell line that was derived by SV40 transformation from an adherent cell enriched population of alveolar macrophages (CRL-2019, ATCC) this cell line expresses CD44s, CD44(v6-v10), Mac-1, integrin $\alpha_V\beta_3$, and integrin subunits β1, β5. MT-2/1 is a thymus-derived macrophage from a Balb/c mouse that was immortalized by infection with retroviral vector (Lutz et al. 1994). It expresses CD44 and integrin $\alpha_V\beta_3$. The AF3.G7 hybridoma, generated by fusing cow insulin-immune C57BL/6 lymph node cells with the BW5147 thymoma line, responds to cow insulin according to IL-2 production (Spinella et al. 1987) and expresses CD44 on its cell surface as judged by FACS analysis with anti-Pgp-1 antibody (clone IM7, Pharmingen).

Osteopontin purification and cleavage. Recombinant GST-osteopontin fusion protein was derived from E. coli, digested with factor Xa and purified by affinity chromatography (Ashkar et al. 1993). Osteopontin was purified to homogeneity as determined by N-terminal sequencing. The native osteopontin used in this study is a full-length protein that is O-glycosylated and highly sialylated, free of sulfate or N-glycosylation and contains 15-17 phosphate residues. Thrombin cleavage of osteopontin was accomplished by human thrombin (Sigma Chemicals). Native osteopontin was purified from conditioned media of MC3T3.C6, a cell clone derived from MC3T3E1 osteoblast like cell line that was selected for over production of OPN. Briefly, MC3T3.C6 was grown in media consisting of DME/H12 supplemented with pyruvate, insulin, transferrin, selenium and ethanol amine in a humidified atmosphere of 5% CO_2 at 37°C. Conditioned media from confluent cultures were collected every 24 h. and stored frozen until processed. 1 liter of conditioned media

was concentrated, into PBS, to 100 ml using Millipore tangential flow system (membrane cut off of 10,000 daltons). The concentrated protein solution was applied to Millipore LC100 equipped with a DEAE-Memsep 1000 cartridge. The cartridge was developed with a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer, pH 7.4. The major Osteopontin peak eluted at 0.26 M salt.

Osteopontin containing fractions were pooled concentrated by ultrafiltration (Filtron 10,000 cut off) and applied to a chromatofocusing mono P column (Pharmacia) at pH 8.2. After washing, the column was developed with polybuffer 74 (Pharmacia). The major Osteopontin containing fractions eluted from the monobeads at pH 4.6. The protein was judged pure by several criteria, including SDS electrophoresis and amino acid sequence analysis (N-terminal and internal peptide analysis). Mass spectroscopic analysis revealed a broad peak centered around a mass of 35,400 as expected from a highly modified protein. The protein was highly phosphorylated (11 mols of phosphate/mol of protein) was O-glycosylated but not N-glycosylated and did not contain any measurable sulfate.

Chemotaxis. Directed migration of cells was determined in multi-well chemotaxis chambers as described (Moses et al. 1990; Weber et al. 1996). Briefly, two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5 μg fibronectin. 2 X 10^5 cells were added to the upper chamber and incubated at 37°C in the presence or absence of osteopontin in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically.

Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. All assays were done in triplicates and are reported as mean ± standard deviation.

Analysis of Signal Transduction. Signal transduction mechanisms were initially examined through the use of specific chemical inhibitors at the following final concentrations: 50 mM for cycloheximide, PKA inhibitors H89 at 1 mM and, H7 at 20 μM, Inhibitors of PI pathway Wortmannin at 10 nM, Tyrosine kinase inhibitors genistein at 25 μM, PKC inhibitor chelerythrine at 20 μM, Casein Kinase II inhibitor quercetin at 6 mM . In all experiments using these compounds 2 x 10^6 macrophages were preincubated for 0.5 hours with the inhibitors before start of the experiment. Cell viability was determined by trypan blue exclusion on cell samples before and after the termination of the experiments. Cell viability in all reported experiments was > 95%. Microfilament disruption was carried out by preincubation of the cell cultures for one hour in 50 μM cytochalasin D. Microtubule dissociation was carried out by pre incubation of the cultures for 6 hours in 1 μM colchicine. All compounds were suspended in either DMSO or absolute ethanol and were added to the culture media at 1:1000 dilution. Controls were carried out with the corresponding vehicle. In separate experiments in which protein kinase C and protein kinase A were chemically activated 50 ng/ml phorbol 12-myristate 13-acetate and 10-5 M of forskolin were used respectively. In these experiments treatments were for 2 hours.

Dephosphorylation after ligation of CD44 by osteopontin. AF3.G7 cells were lysed in 0.1% Triton X 100 buffer containing 25 mM potassium phosphate buffer, pH 7.4, 4 mM EDTA, 10 mM sodium chloride, 5 mM magnesium chloride, 10 mM iodoacetamide,

0.025% (w/v) sodium azide, 0.2 U/ml aprotinin, 20 mg/ml pepstatin A, 1 mM phenyl-methyl-sulfonyl-fluoride, and 1 mM sodium orthovandate, centrifuged at 100,000 g before the supernatant was filtered and loaded onto the indicated affinity resins overnight. After extensive washing the bound protein was eluted with 3 M NaSCN and salt was removed in Centricon^{R} filter units and Excellulose GF-5 detergent removal columns (Pierce) followed by concentration in Microsep 10K filters (Filtron).

Activation of SHP-1. Activation of the phosphatase SHP-1 was assayed by (...).

RESULTS

Agents that disrupt the cytoskeleton as well as tyrosine kinase inhibitors diminish OPN mediated chemotaxis, haptotaxis and spreading. Osteopontin dependent chemotaxis, but not haptotaxis and spreading, is inhibited by pertussis toxin but not by inhibitors of protein kinase C or A (Table 1). Therefore, CD44 signaling proceeds via G-protein activation. The concentrations of inhibitors used are considered to be non-toxic and the selective inhibition of macrophage responses argues against a toxic effect of these inhibitors.

We had noted earlier that purification of CD44 from cell lysates on osteopontin affinity columns was associated with diminished phosphorylation compared to CD44 purified on anti-CD44 (KM81) immunoaffinity columns. Moreover, co-incubation of ^{32}P-labelled CD44 and various combinations of cold recombinant osteopontin resulted in dose-dependent dephosphorylation of purified CD44. By contrast, coincubation of the same preparation of osteopontin with ^{32}P-labelled casein had no effect. Moreover, incubation of osteopontin with ^{32}P-labelled membrane proteins from a CD44-negative cell line (A31) did not induce loss of radiophosphate. Dephosphorylation of CD44 was not due to a contaminating phosphatase in the recombinant osteopontin preparation, since neither alkaline phosphatase nor acid phosphatase activity (both of which are expressed in E. coli) were detected (Figure 1).

The candidate CD44-associated phosphatase to mediate this activity is SHP-1. The C-terminal fragment of osteopontin, which ligates CD44 also mediates phosphorylation of

SHP-1, a process that causes activation of this phosphatase. A substrate for SHP-1 is phosphatidylinositol 3-kinase. In fact, phosphorylation of SHP-1 is accompanied by reduced phosphate on phosphatidylinositol 3-kinase (Figure 2).

These experiments map CD44 signal transduction to a pathway involving G-protein and SHP-1 activation followed by inactivation of PI 3-kinase.

CD44 in tumor cells may be essential for metastasis formation. We tested whether the same signal transduction pathway is active in various cancer cells. Activation of SHP-1 after ligation by osteopontin occurs in MDA-MB-231, but not MDA-MB-330 or MDA-MB-457 cells. It is also observed in a $CD44^+$, ER^+ variant of MCF-7 cells (not in a CD44-, ER- variant). LNCap prostate cancer, MES-SA/DX5 ovarian cancer (CD44+, resistant to tamoxifen, taxol, cisplatin), and CD44+ melanoma **(which one?? Correct list??)** engage this mechanism. In contrast, the non-metastatic SW403 colorectal does not (Figure 3). Activation of SHP-1 after engagement by osteopontin correlates with chemotaxis to this ligand (data not shown).

DISCUSSION

The in vitro interaction between osteopontin and CD44 leads to dephosphorylation of CD44 receptor associated proteins, either as a direct consequence of this bimolecular interaction or as a result of a CD44-associated enzyme which is activated by osteopontin binding. In either case, the observation that the dephosphorylation which accompanies osteopontin binding to CD44 is not detectable following ligation by HA (not shown) may be relevant to the different effects of the two ligands on cell behavior. Our observations suggest that the interaction between CD44 and its two ligands can elicit distinct functional consequences due to differential activation of the SHP-1 phosphatase. Gradients of osteopontin induce CD44-dependent chemotaxis while HA does not. Conversely, HA induces CD44-dependent cell aggregation whereas osteopontin does not (Weber et al. 1996).

The molecular basis for the association between CD44 and SHP-1 is uncertain. In particular, we do not know whether SHP-1 binds to the cytoplasmic tail of CD44 directly or through association with adaptor molecules. There are three possible ways in which SHP-1 may be associated with CD44. 1) Here we have shown activation of a G-protein to be involved in osteopontin dependent CD44 engagement and SHP-1 may be a substrate for G-proteins. 2) Src can be associated with CD44 (Bourguignon et al. 2000) and Src phosphorylates SHP-1. 3) p56lck (which may also bind to CD44) can phosphorylate and activate SHP-1 (Binstadt et al. 1996).

There are currently four candidate pathways to account for the pro-migratory and anti-adhesive effects of SHP-1. 1) SHP-1 associates with phosphatidylinositol 3-kinase (PI

3-kinase) in macrophages and PI 3-kinase tyrosine phosphorylation and kinase activity are elevated in SHP-1-deficient macrophages. Furthermore, treatment of SHP-1 deficient macrophages with the PI 3-kinase inhibitor wortmannin results in detachment from integrin contacts (Roach et al. 1998). Therefore, SHP-1 decreases phosphatidylinositol 3-kinase activity by dephosphorylation, a function necessary for de-adhesion. 2) CD44 may associate in the cell membrane with a linker protein that connects it to the cytoplasm. This linker protein rather than CD44 itself may be the substrate for tyrosine phosphorylation and dephosphorylation. Candidate linker proteins are the integrins β_1, β_3, and β_5 since all possess potential tyrosine phosphorylation sites (Hynes et al. 1992). Alternatively, SHP-1 could dephosphorylate PECAM-1 which mediates reversal of PECAM-1 induced inhibition of integrin β_1-induced cell migration (Lu et al. 1996). This may be relevant since integrin β_1 has been found to associate with CD44, which implies the possibility that osteopontin may co-ligate both chains (Katagiri et al. 1999). 3) The NF-2 protein merlin colocalizes with ezrin and CD44 (Sainio et al. 1997). Merlin is phosphorylated on serine/threonine residues. Furthermore, loss of adhesion results in a nearly complete dephosphorylation of Merlin, which is reversed upon re-plating of cells, suggesting Merlin phosphorylation may be responsive to cell spreading (Shaw et al. 1998). Dephosphorylation of merlin may reflect the action of SHP-1. 4) SHP-1 may dephosphorylate p120 catenin (Keilhack et al. 2000).

SHP-1 is a cytoplasmic protein tyrosine phosphatase that contains two Src-homology 2 (SH2) domains. A regulatory role of SHP-1 in the immune system has been strongly implicated by the phenotype of knockout mice and motheaten mice with a natural mutation in the Shp-1 locus. Affected mice are characterized by leukocyte hypersensitivity,

deregulated macrophage and mast cell functions and excessive erythropoiesis. They die prematurely, primarily due to macrophage and neutrophil accumulation in the lung and other tissues (Tsui/Tsui 1994). The leukocyte infiltrate in the lung results in destruction of bronchi, thickening of the alveolar septa, and granuloma formation. SHP-1-macrophages maintain tightly adherent surfaces through integrin-mediated contacts due to failure to regulate de-adhesion. SHP-1 associates and can be co-immunoprecipitated with phosphatidylinositol 3-kinase (PI 3-kinase) in macrophages and PI 3-kinase tyrosine phosphorylation and kinase activity are elevated in SHP-1-deficient macrophages. Furthermore, treatment of SHP-1- deficient macrophages with the PI 3-kinase inhibitor wortmannin results in the detachment of macrophages from integrin contacts **(ref.??)**. Therefore, SHP-1 decreases PI 3-kinase activity, a function necessary for de-adhesion.

(G-proteins may inhibit IL-10 expression) Ligation of CD44 by osteopontin leads to inhibition of IL-10 secretion. Consistently, motheaten viable mice with a defect in SHP-1 display elevated levels of this cytokine (Khaled et al. 1998).

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LEGENDS TO TABLES & FIGURES

Table 1. Migratory Respose of Macrophages to Osteopontin. Chemotactic activity of osteopontin and the C-terminal fragment of osteopontin Chemotactic activity of osteopontin fragments after thrombin degradation, separation and purification was tested in a modified Boyden chamber (Weber et al. 1996). Purified natural osteopontin exerted chemotactic activity. The C-terminal (CT) thrombin cleavage product (pos 154-294) but not the N-terminal (NT) thrombin cleavage product (pos 1-153) of osteopontin also mediated chemotaxis. Inhibition of migration by osteopontin fragments or modulators of receptor interaction.

Figure 1: Dephosphorylation of CD44 by osteopontin in vitro. A,B) Western blot analysis of osteopontin binding material. Material from AF3.G7 T-cell lysate (described in Weber et al. 1996) bound to osteopontin columns (GST-Opn immobilized on Sepharose 4B with GST Sepharose 4B as precolumn), material bound to anti-CD44 columns (immobilized anti-mouse CD44 antibody KM81, ATCC clone TIB 241, with rat-IgG Sepharose 4B as precolumn), and AF3.G7 whole cell lysate were separated on SDS-PAGE (A) and on native PAGE, in Tris-CAPS (pH 9.4), followed by transfer to nitrocellulose and Western blotting with anti-CD44 antibody (KM81) (B). The bands visualized by Western blotting of osteopontin binding material and antibody-purified CD44 co-migrated. Non-specific binding was excluded since stripping and reprobing the membrane with an irrelevant antibody did not reveal a detectable band. C) Phosphotyrosine content of CD44 and osteopontin receptor. Anti-phosphotyrosine antibody recognizes CD44 but not the osteopontin receptor. Western blotting with anti-phosphotyrosine antibody 4G10 was

performed on eluted material bound to recombinant osteopontin columns, anti-CD44 binding material and AF3.G7 whole cell lysate after stripping and reprobing membranes. D) Dephosphorylation of CD44 by osteopontin. AF3.G7 cells ($90x10^6$) were labelled with 2.5 mCi $H_3{}^{32}PO_4$ for 4 hours before lysis and purification of CD44 by immunoaffinity chromatography. After desalting, 10,000 cpm of eluted material was incubated with the indicated concentrations of cold recombinant osteopontin in 20 mM phosphate buffer, pH 7.4, 150 mM sodium chloride, 10 mM magnesium chloride in 50 µl for 30 min at 37°C before separation of 25 µl by SDS-PAGE and autoradiography. Recombinant osteopontin depletes radiolabelled phosphate from anti-CD44 binding material in a dose dependent fashion.

Figure 2: Activation of SHP-1 and inhibition of PI 3-kinase after ligation of CD44 by osteopontin. MH-S cells were incubated with osteopontin. Cells were lysed, SHP-1 or PI 3-kinase were immunoprecipitated with specific antibodies, and their levels of tyrosine phosphorylation were determined by Western blotting. Fragments of osteopontin that ligate integrin $\alpha_V\beta_3$ induce PI 3-kinase but not SHP-1. In contrast, fragments that engage CD44 mediate SHP-1 activation which then dephosphorylates PI 3-kinase. NK10 = N-terminal 10 kD peptide reaching from aa 70 (QETLPSN) to the thrombin cleavage site at aa153. This fragment is the smallest identified domain that transduces signals through integrin $\alpha_V\beta_3$. OPN-NT and OPN-CT = N-terminal and C-terminal thrombin cleavage fragments of osteopontin respectively. OPN = Purified native osteopontin. DeP-OPN = dephosphorylated native osteopontin.

Figure 3: Activation of SHP-1 in various cancer cells following ligation by osteopontin. A = Sw403 colon cancer (CD44v-, CD44s+); B= MDA-MB-453 (non-metastatic CD44v-); C= Macrophages (thioglycol-elicited); D= MDA-MB-330 (CD44-); E= MDA-MB-231 (CD44+); F= MDA-MB-330 (CD44 v3-v10); G=MDA-MB330 (CD44 v3-v6); H= MCF-7.

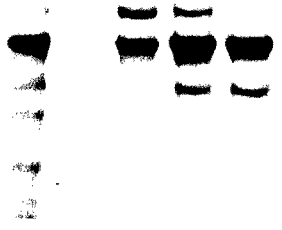
	OPN	OPN-CT
Control	1.0 ± 0.3	1.0 ± 0.2
Osteopontin	13.3 ± 1.9 *	9.6 ± 1.9 *
+ GRGDS (1mM)	10.6 ± 1.3 *	7.7 ± 1.6 *
+ anti CD44	7.8 ± 0.7	4.6 ± 0.7
+ wortmannin (10 mM)	12.6 ± 2.5 *	10.5 ± 1.6 *
+ chelerythrine (20 μM)	3.1 ± 0.5	1.9 ± 0.8
+ genistein (25 μM)	6.6 ± 1.1	4.3 ± 0.7
+ pertussis toxin	2.3 ± 0.2	1.6 ± 0.3
+ H 7 (20 μM)	14.1 ± 2.7 *	8.7 ± 1.2 *
+ cytochalasin D (1 μM)	1.8 ± 0.9	2.2 ± 0.3

* $p < 0.05$

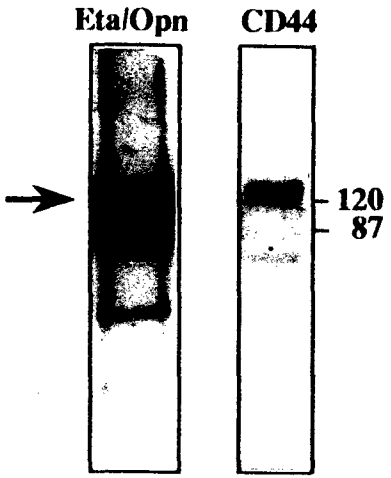
A = Sw403 colon cancer (CD44v-, CD44s+)
B= MDA-MB-453 (non met CD44v-)
C= Macrophages (thioglycol-illicited)
D= Mda-MBA-330 (CD44-)
E= MDA-MB-231 (CD44+)
F= MDA-MB-330 (CD44 V3-v10)
G=MDa-MB330(V3-V6)
H= MCF-7

A B C D E F G H

SHP →



A.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CD44
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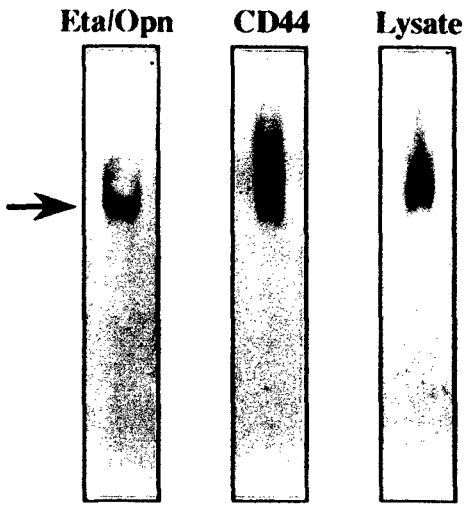
87

B.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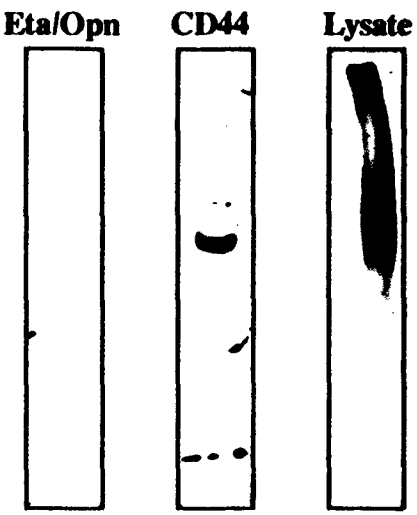
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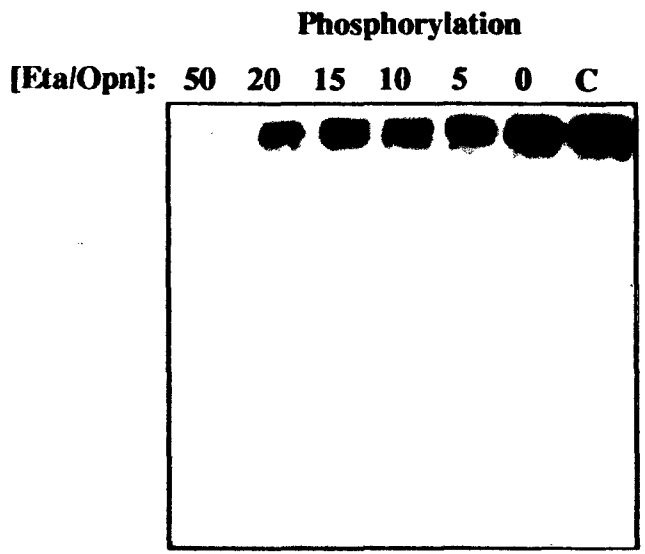
CD44
Lysate

D.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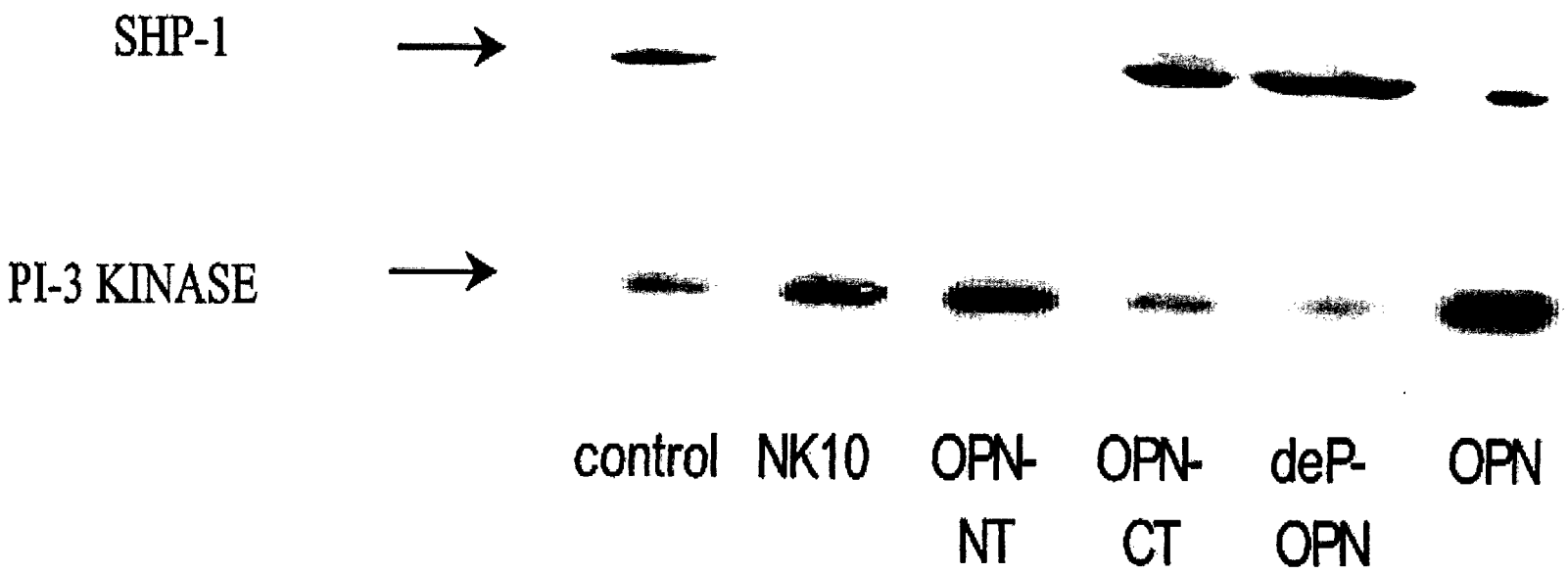
SHP-1
PI-3 KINASE
control
NK10
OPN-NT
OPN-CT
deP-OPN
OPN

Two Distinct Signal Transduction Pathways Associated with Ligation of Integrin $\alpha_v\beta_3$ by Osteopontin

Key terms: Osteopontin, phosphorylation, integrins, macrophages, cancer, cytokines, metalloproteases

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ABSTRACT

The cytokine osteopontin has multiple domains that engage the two prime receptors, CD44 and integrin $\alpha_V\beta_3$, in a division of labor. Engagement of integrin receptors on macrophages or malignant tumors by an N-terminal osteopontin domain induces haptotaxis followed by cell spreading and cytokine secretion. While haptotaxis is resistant to the wotmannin reagent, to the G-protein inhibitor pertussis toxin, and to inhibitors of protein kinase A, it is sensitive to the protein kinase C inhibitor chelerythrine. Induction of protein kinase C activity is associated with ligation of integrin $\alpha_V\beta_3$ by osteopontin. After spreading of the cells on osteopontin, a second signal transduction component is added through activation of phosphatidylinositol-3 kinase which leads to downstream activation of Stats and to secretion of cytokines. Therefore, ligation of integrin $\alpha_V\beta_3$ on macrophages by osteopontin proceeds in a two step process in which haptotaxis is mediated by a protein kinase C containing pathway; at the highest concentration of ligand, haptotaxis is followed by cell adhesion and spreading which engages a second, phosphatidylinositol 3-kinase containing pathway. The recruitment of additional signaling components after spreading also provides evidence for the dependence of signal transduction on cell morphology.

INTRODUCTION

Osteopontin is an important immunomodulatory cytokine that exerts its effects through a division of labor between two domains which engage distinct receptors. While ligation of CD44 by a C-terminal fragment of osteopontin leads to chemotaxis, interaction between an N-terminal osteopontin domain and the integrin $\alpha_V\beta_3$ mediates haptotaxis followed by spreading and cytokine secretion (Weber et al. 2000, submitted for publication). Necessarily, the two functions of the integrin receptor must be mediated by distinct intracellular signals, however, the molecular mechanisms activating the complementary response phenotypes are incompletely understood.

A proximal mediator of osteopontin dependent signal transduction through integrin receptors is the 125 kD focal adhesion kinase (Hruska et al. 1995) which associates with integrin $\alpha_V\beta_3$ and, in synergy with the cytoplasmic tail of integrin α_V, activates pp60c-Src (Chellaiah et al. 1996). Two downstream processes ensue which both modulate the cytoskeleton. Tensin and paxillin may be activated by p125 FAK and pp60c-Src (Lopez et al. OPN book, pp 324-326). Src also activates phosphoinositide 3-hydroxyl kinase by tyrosine phosphorylation of the Src homology 2 domain in the p85 subunit (Hruska et al. OPN book). PI 3-kinase may regulate the arrangement of actin filaments through gelsolin in a process that is inhibitable by wortmannin (Chellaiah/Hruska 1996). While both pathways have been associated with cell motility and spreading, their individual contributions to these processes are not known. Here we analyze differential signal transduction after ligation of integrin $\alpha_V\beta_3$ by osteopontin which leads to either haptotaxis or spreading and cytokine secretion.

MATERIALS AND METHODS

Cell lines. MH-S is a macrophage cell line that was derived by SV40 transformation from an adherent cell enriched population of alveolar macrophages (CRL-2019, ATCC) this cell line expresses CD44s, CD44(v6-v10), Mac-1, integrin $\alpha_V\beta_3$, and integrin subunits $\beta1$, $\beta5$. MT-2/1 is a thymus-derived macrophage from a Balb/c mouse that was immortalized by infection with retroviral vector (Lutz et al. 1994). It expresses CD44 and integrin $\alpha_V\beta_3$. Several tumor cell lines were analyzed, MDA-MB-231, an MCF-7 (CD44+, ER+) variant, LNCaP, and MES-SA/DX5 ovarian cancer.

Osteopontin purification and cleavage. Recombinant GST-osteopontin fusion protein was derived from E. coli, digested with factor Xa and purified by affinity chromatography, as described (Ashkar et al. 1993a,b). Osteopontin was purified to homogeneity as determined by N-terminal sequencing. The native osteopontin used in this study is a full-length protein that is O-glycosylated and highly sialylated, free of sulfate or N-glycosylation and contains 15-17 phosphate residues. Thrombin cleavage and phosphorylation of osteopontin was accomplished by human thrombin (Sigma Chemicals), Golgi kinases or casein kinase II as described (Ashkar et al. 1993a; Salih et al. 1996a,b). Native osteopontin was purified from conditioned media of MC3T3.C6, a cell clone derived from MC3T3E1 osteoblast like cell line that was selected for overproduction of osteopontin. Briefly, MC3T3.C6 was grown in media consisting of DME/H12 supplemented with pyruvate, insulin, transferrin, selenium and ethanol amine in a humidified atmosphere of 5% CO2 at 37°C. Conditioned media from confluent cultures were collected every 24 h. and stored frozen until processed. 1 liter of conditioned media was concentrated, into PBS, to 100 ml using Millipore tangential flow

system (membrane cut off of 10,000 daltons). The concentrated protein solution was applied to Millipore LC100 equipped with a DEAE-Memsep 1000 cartridge. The cartridge was developed with a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer, pH 7.4. The major osteopontin peak eluted at 0.26 M salt. osteopontin containing fractions were pooled concentrated by ultrafiltration (Filtron 10,000 cut off) and applied to a chromatofocusing mono P column (Pharmacia) at pH 8.2. After washing, the column was developed with polybuffer 74 (Pharmacia). The major osteopontin containing fractions eluted from the monobeads at pH 4.6. The protein was judged pure by SDS electrophoresis and amino acid sequence analysis (N-terminal and internal peptide analysis). Mass spectroscopy revealed a broad peak centered around a mass of 35,400 as expected from a highly modified protein. The protein was extensively phosphorylated (11 mols of phosphate/mol of protein), was O-glycosylated but not N-glycosylated, and did not contain any measurable sulfate.

Modulation of the phosphorylation status of osteopontin. 5 mg of osteopontin was dephosphorylated using 6 units (60 units/mg) type II potato acid phosphatase (Sigma # p3752) in 20 mM phosphate buffer pH 4.8 and incubated at 37oC for 2 h. After adjusting the pH to 8.2, the dephosphorylated protein was applied to a chromatofocusing column. The protein was eluted with polybuffer as described. Multiple peaks containing OPN were detected. The major peak eluted with a pH of 5.1. Amino acid analysis of the protein revealed a phosphate content of less than 1mol/mol protein. In another approach, 5 mg of GST-OPN was incubated at 37°C with 10 μg of golgi kinases in phosphate buffer containing 10mM ATP, 10 mM $MgCl_2$, 1mM $MnCl_2$,0.1 mM $CaCl_2$, 1μM cGMP, 1 mM

pyrophosphate, 1 mM diacylglycerol, and 1 mM NaF. After 2 h, the phosphorylated protein was applied to a GSH-Sepharose column. The resin was washed with 5 volumes of PBS. The rOPN was eluted from the GSH-Beads by incubating the beads with 100 U of factor Xa as described. After 2 h at 37oC, the released rOPN was applied to a chromatofocusing column. The phosphorylated rOPN was eluted from the resin with polybuffer 74 as described. The major peak eluted with pH 4.6. Phopho-amino acid analysis of the recovered protein revealed a phosphoserine content of 16 mol of phosphate/mol protein and 0.8 mols of phosphotherione/mol protein.

Haptotaxis. Crawling of MH-S or MT-2 monocytic cell lines to osteopontin or fragments of osteopontin was assayed using a Boyden chamber. The lower surface or both sides of polycarbonate filters with 8 μm pore size were coated with 5 μg of osteopontin. This concentration is saturating since osteopontin bound to filters with an EC_{50} of around 3.2 pM. 2 X 10^5 cells were added to the upper chamber, and incubated at 37°C in the absence of soluble factors in the lower chamber. After 4 h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells that had migrated to the lower surface were counted under a microscope. All assays were done in triplicates and are reported as mean ± standard deviation.

Cell spreading. Twenty-four well plates were coated with 10 μg/ml protein in PBS overnight at 4°C. Non-specific sites were blocked with 10 mg/ml BSA in PBS for 60 minutes. To preserve the integrity of adhesion receptors proliferating MH-S cells were collected using non-enzymatic cell dissociation solution (Sigma BioSciences) before

resuspension in sterile Ca^{2+} and Mg^{2+}-free PBS and addition of $5x10^3$ cells to each well and incubation at 37°C in 95% O_2/5% CO_2 for 1 hour. After removal of medium and loosely attached cells with sterile PBS, adherent cells were fixed with 1% paraformaldehyde, 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 hour, followed by staining with Hematoxylin solution for 1 hour, washing with distilled deionized water and staining with 0.1% Toluidine Blue overnight. Photomicrographs of 4-5 non-overlapping fields were taken at 100X magnification for cell counting (Olympus microscope). Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area. In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Cytokine secretion. The production of cytokines was determined using commercial ELISA kits. IL-10 and IL-12 were measured with a kit from R&D Systems. ELISA kits for TNFα and $TGF\beta_1$ came from Research Diagnostics Inc., Flanders NJ.

Analysis of signal transduction. Signal transduction mechanisms were initially examined through the use of specific chemical inhibitors at the following final concentrations: 50 mM for cycloheximide, PKA inhibitor H89 at 1 mM and, H7 at 20 μM, the inhibitor of the PI pathway, Wortmannin, at 10 nM, the tyrosine kinase inhibitor genistein at 25 μM, the PKC inhibitor chelerythrine at 20 μM, and the casein kinase II inhibitor quercetin at 6 mM . In all experiments using these compounds 2×10^6 macrophages were preincubated for 0.5 hours with the inhibitors before start of the experiment. Cell viability was determined by

trypan blue exclusion on cell samples before and after the termination of the experiments. Cell viability in all reported experiments was > 95%. Microfilament disruption was carried out by preincubation of the cell cultures for one hour in 50 μM cytochalasin D. Microtubule dissociation was carried out by preincubation of the cultures for 6 hours in 1 μM colchicine. All compounds were suspended in either DMSO or absolute ethanol and were added to the culture media at 1:1000 dilution. Controls were carried out with the corresponding vehicle. In separate experiments in which protein kinase C and protein kinase A were chemically activated 50 ng/ml phorbol 12-myristate 13-acetate and 10^{-5} M of forskolin were used respectively. In these experiments treatments were for 2 hours.

Activation of protein kinase C. $1x10^6$ macrophages were treated with 10 μg/ml osteopontin. The cells were harvested and lysed and the activity of protein kinase C was measured with a kit obtained from Panvera. This assay distinguishes isoforms of protein kinase C according to their calcium dependence. We performed two separate experiments with duplicate samples.

Activation of phosphatidylinositol-3 kinase. Macrophages were lysed in 1 ml buffer A (10 mM Tris-HCl buffer, pH 7.2, containing 300 mM sucrose, 100 mM KCl, 1% Triton X 100 5 mM $MgCl_2$, 10 mM EGTA, 0.1 mM sodium orthovanadate, 0.1 M ε-amino-n-caproic acid, 5 mM benzamidine, 1 mM p-hydroxymercuribenzoate, 5 mg/l pepstatin, 1 mg/l leupeptin) at 4°C. After 10 min, fractions containing 2 mg protein were pre-cleared by incubation with 100 μl of insoluble protein A at 4°C for 1 h. After centrifugation at 5000 x g for 10 min., the resultant supernatant was incubated with 0.1 mg of rabbit polyclonal

antibody raised against the p85a subunit of PI-3-Kinase (Upstate Biotechnology, Lake Placid, N.Y.). The immune complexes were collected by incubation with 10 µl insoluble protein A for 1 h at 4°C followed by centrifugation at 5000 x g for 10 min. The protein A-immunocomplexes were washed 5x with lysis buffer, then once with 20 mM Tris-HCl. The immune complexes were released from the protein A beads by boiling the beads in 20 ml of SDS sample buffer containing 0.1% fresh 2-mercaptoethanol. The samples were resolved by on an 8% SDS-polyacrylamide gel and then transferred onto ECL-membrane by semi-dry blotting as described by the manufacturer. Phosphorylation of PI-3-kinase was assessed by probing the membranes with anti-phosphotyrosine.

Activation of Stats. $5x10^5$ cells were grown on osteopontin (10 µg/ml) in 0.5 ml DMEM for 2 h to allow spreading. Cells were lysed and immunoprecipitated with antibodies to various Stats. After separation on 10% SDS-PAGE, the immunoprecipitates were probed with anti-phosphotyrosine antibody.

RESULTS

We have demonstrated that osteopontin exerts its effects on macrophages through a division of labor between a C-terminal domain that ligates CD44 and an N-terminal domain that may engage integrin $\alpha_V\beta_3$ after phosphorylation or proteolytic cleavage of the ligand (Ashkar et al. 2000; Weber et al. 2000, submitted for publication). Binding of osteopontin to its integrin receptor sequentially leads to haptotaxis followed by spreading and cytokine secretion. In order to delineate candidate signaling pathways we tested the effect of several protein kinase inhibitors and cytoskeletal disrupting agents on haptotaxis, cell spreading, and activation of macrophages mediated by osteopontin. Agents that disrupt the cytoskeleton as well as tyrosine kinase inhibitors at non-toxic concentrations as judged by Trypan blue exclusion inhibit osteopontin mediated chemotaxis (a CD44 mediated effect), haptotaxis and spreading. Haptotaxis is not affected by pertussis toxin or the protein kinase A inhibitor H7 or by the Wortmannin reagent but is inhibited by the protein kinase C inhibitor chelerythrine at non-toxic concentrations as judged by Trypan blue exclusion. Furthermore, the selective inhibition of macrophage responses argues against a toxic effect of these inhibitors (Table 1). Protein kianse C activity is induced after receptor engagement by osteopontin or its N-terminal domain, but not by the C-terminal fragment (Figure 1). While the γ form of PKC is insensitive to osteopontin, the η isoform is strongly induced. Therefore, an early step in integrin mediated signaling is the activation of protein kinase C.

After haptotaxis on osteopontin, when a maximal concentration of the ligand is reached, the cells spread. While this process is also mediated by integrin $\alpha_V\beta_3$, the more homogeneous occupation of receptors by osteopontin causes a change in cell morphology

and response phenotype. At this point, additional signal transduction mechanisms beside activation of protein kinase C are invoked. Like haptotaxis, spreading of macrophages on osteopontin is inhibited by chelerythrine (Table 1B). Furthermore, phosphatidylinositol-3-kinase is activated as judged by both, inhibition of spreading with Wortmannin reagent and by direct measurement of PI-3 kinase phosphorylation in spreading but not haptotaxing cells (Figure 2).

Upon spreading, macrophages are activated to secrete various cytokines, including IL-12, TNF-α and TGF-β (ref. Weber et al. 2000 submitted). Like spreading, cytokine secretion is inhibitable by inhibitors of the cytoskeleton (genistein), protein kinase C (chelerythrine), and PI-3 kinase (Wortmannin), but not by inhibitors of G-proteins (pertussis toxin), or protein kinase A (H7) (Figure 4). This suggests that the induction of both integrin $\alpha_V\beta_3$ associated pathways is needed to induce cytokine secretion.

In sum, these data indicate that integrin-dependent haptotaxis is mediated by a pathway involving protein kinase C. Once a cell has spread, phosphatidylinositol signaling is integrated as a second component into integrin-dependent signal transduction. This leads to macrophage activation, including the secretion of cytokines.

DISCUSSION

Earlier studies have identified a role for PKC in cell crawling. Treatment of the colon carcinoma cell line HT29-D4 with PMA increased the rate of cell spreading and induced the migration of these cells towards purified matrix proteins in Boyden chamber-based haptotaxis assays. HT29-D4 cell haptotaxis was a direct consequence of PKC activation and not secondary to quantitative or qualitative changes in the cell surface integrins (Rigot et al. 1998). In crawling T cells, triggered via cross-linking of integrin LFA-1, two PKC isoenzymes, β(I) and δ, are targeted to the cytoskeleton with specific localization corresponding to the microtubule-organizing center and microtubules. Cells of a PKC-β-deficient clone derived from the parental PKCβ-expressing T cell line can neither crawl nor develop a polarized microtubule array upon integrin cross-linking. However, their adhesion and formation of actin-based pseudopodia remain unaffected (Volkov et al. 1998).

Integrins may be associated with multiple signal transduction pathways, engagement of which is regulated by cell morphology. Similar to osteopontin, thrombospondin may induce various cellular responses, including chemotaxis and haptotaxis. The antiproliferative activity of thrombospondin-1 is mediated by a different signal transduction pathway than those mediating motility responses to the same protein. Thrombospondin-1-mediated chemotaxis is partially dependent on a pertussis toxin-sensitive G-binding protein, whereas haptotaxis is not (Guo et al. 1998). Monoclonal antibody C6.7, which binds to the carboxy-terminal region of thrombospondin, dose-dependently inhibits haptotaxis without affecting chemotaxis by human melanoma cells. In contrast, monoclonal antibody A2.5, heparin, and fucoidan, which bind to the amino-terminal heparin-binding domain of

thrombospondin, inhibit chemotaxis but not haptotaxis. Monoclonal antibody A6.1 directed against the internal core region of thrombospondin has no significant effect on either haptotaxis or chemotaxis. The 140 kD fragment of thrombospondin lacking the heparin-binding amino-terminal region retains the property to fully mediate haptotaxis but not chemotaxis, however, when the carboxy-terminal region containing the C6.7-binding site is removed, the resulting 120 kD fragment (which retains the RGDA sequence) looses the ability to induce haptotaxis. Separate structural domains of thrombospondin are therefore required for tumor cell haptotaxis vs. chemotaxis (Taraboletti et al. 1987).

ACKNOWLEDGEMENTS

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LEGENDS TO TABLES & FIGURES

Table 1: Signaling associated with haptotaxis and spreading. Use of inhibitors to delineate signaling mechanisms that induce cell crawling or spreading. MH-S monocytic cells (10^3/well) were incubated in 96-well plates that had been coated with 10 μg/ml of the indicated ligands for 1 hr, fixed, and stained with toluidine blue. A) Haptotactic activity of osteopontin and the N-terminal fragment of osteopontin. Migration of MH-S across polycarbonated filters in response to bound osteopontin. Coated filters were placed in a Boyden chamber with the coated side up (top row), or down (left column). Or to both sides (diagonal). Data are expressed as migratory index (cells migrating in response to osteopontin/cells migration in response to buffer). Values are expressed as mean ± SEM. Monocyte migration was mainly directional (i.e., the cells responded to a positive gradient of bound osteopontin), and thus haptotactic. Values above and along the diagonal were also greater than the buffer control, but less than that below the diagonal, indicating that a minor component of monocyte migration to bound osteopontin was random. B) **(effects of inhibitors on spreading, need to include spreading data)**

Figure 1: Induction of protein kinase C by osteopontin. $1x10^6$ macrophages were treated with 10 μg/ml osteopontin. The cells were harvested and lysed and the activity of protein kinase C was measured with a kit obtained from Panvera. This assay distinguishes isoforms of protein kinase C according to their calcium dependence. We performed two separate experiments with duplicate samples.

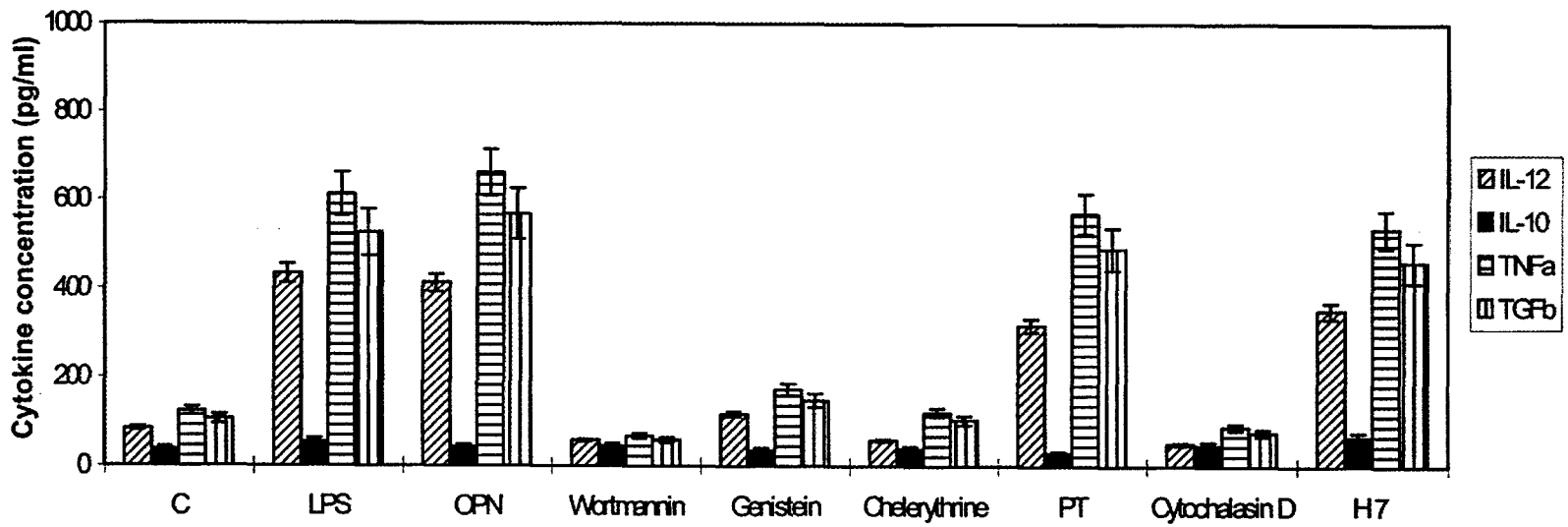
Figure 2: Measurement of PI-3 and spreading. Native osteopontin or its RGD binding domain NK10, but not the C-terminal fragment or dephosphorylated osteopontin, induce cell spreading and phosphorylation of PI-3 kinase.

Figure 3: Regulation of Cytokine Expression by OPN. Macrophages were cultured on the indicated ligand for 12 h in defined medium. Secretion of TNFα, TGFβ, the Th1 cytokine IL-12, and the Th2 cytokine IL-10 were determined by commercial ELISA kits. Effect of signaling inhibitors on osteopontin mediated activity.

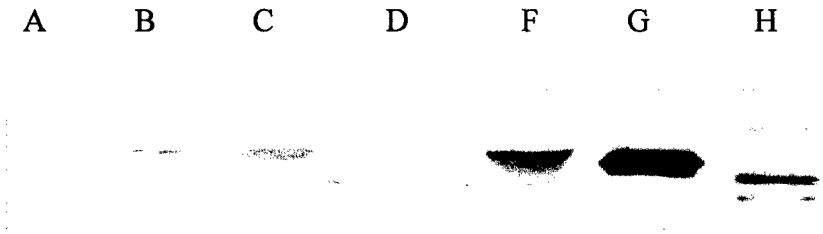
	OPN	OPN-NT
Control	1.0 ± 0.3	0.6 ± 0.2
Osteopontin	9.8 ± 0.9 *	6.1 ± 0.7 *
+ GRGDS (1mM)	3.6 ± 1.0	2.1 ± 0.2
+ wortmannin (10 mM)	10.1 ± 2.2 *	8.8 ± 1.3 *
+ chelerythrine (20 μM)	3.3 ± 0.2	1.3 ± 0.1
+ genistein (25 μM)	3.2 ± 0.5	2.0 ± 0.3
+ pertussis toxin	10.6 ± 1.1 *	9.4 ± 1.5 *
+ H 7 (20 μM)	10.8 ± 1.7 *	6.9 ± 2.0 *
+ cytochalasin D (1 μM)	1.1 ± 0.2	2.7 ± 0.1

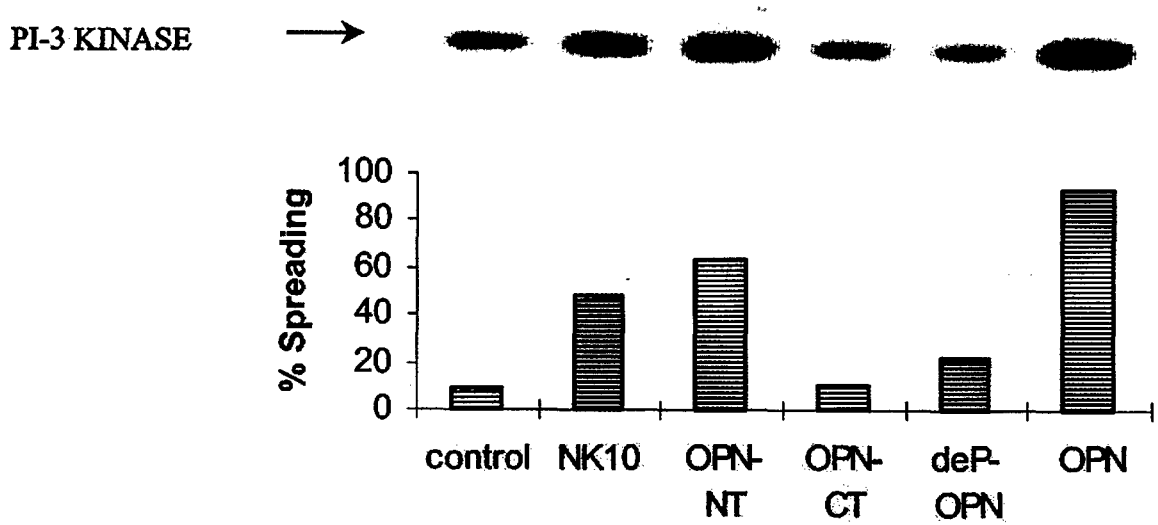
* $p < 0.01$

	Spreading Index	Fold induction of TGFβ
control	1.0 ± 0.18	1.0 ± 0.2
OPN	10.0 ± 1.6	3.8 ± 0.8
OPN-CT	1.7 ± 0.32	0.8 ± 0.17
OPN-NT	10.8 ± 2	3.1 ± 0.7
NT10k	7.1 ± 1.3	2.0 ± 0.4
Wortmannin (10nM)	1.3 ± 0.4	0.8 ± 0.18
Genistein (25 μM)	5.0 ± 1	0.4 ± 0.08
Chelerythrine (20 μM)	0.9 ± 0.16	0.1 ± 0.02
PT	11.0 ± 2	2.6 ± 0.6
Cytochalasin D (1 μM)	0.9 ± 0.18	0.6 ± 0.1
H 7 (20 μM)	10.0 ± 1.1	4.0 ± 0.9


Cytokine concentration (pg/ml)
1000
800
600
400
200
0
C
LPS
OPN
Wortmannin
Genistein
Chelerythrine
PT
Cytochalasin D
H7
IL-12
IL-10
TNFa
TGFb

A = Sw403 colon cancer (CD44v-, CD44s+)
B= MDA-MB-453 (non met CD44v-)
C= Macrophages (thioglycol-illicited)
D= Mda-MBA-330 (CD44-)
F= MDA-MB-330 (CD44 V3-v10)
G=MDa-MB330(V3-V6)
H= MCF-7




PI-3 KINASE
% Spreading
100
80
60
40
20
0
control
NK10
OPN-NT
OPN-CT
deP-OPN
OPN

	- OPN	-OPN	+ OPN	+OPN	
Reaction conditions	PKC η	PKC γ	PKC η	PKC γ	
PS	54	44	768	153	
No calcium	23	-	633	32	

Figure 3 D

	Spreading Index	Fold induction of TGFb
control	1 ± 0.18	1± 0.2
OPN	10 ±1.6	3.8 ±0.8
OPN-CT	1.7 ±0.32	0.8 ±0.17
OPN-NT	10.8 ± 2	3.1 ±0.7
NT10k	7.1 ± 1.3	2± 0.4
Wortmannin (10nM)	1.3 ±0.4	0.8 ± 0.18
Genistein 25 uM)	5 ±1	0.4 ± 0.08
Chelerythrine (20 uM)	0.9 ±0.16	0.1 ± 0.02
PT	11 ± 2	2.6 ± 0.6
Cytochalasin D 1 uM)	0.9 ± 0.18	0.6 ± 0.1
H 7 (20 uM)	10 ± 1.1	4.0 ± 0.9

Figure 3 (c)

Phosphorylated OPN			
	Haptotactic index	Chemotactic index	Spreading index
control	1 ± 0.1	1 ± 0.3	1 ± 0.3
OPN	9.8± 0.9 *	13.3 ±1.9*	10 ± 2.1*
OPN-NT	1.8 ± 0.7	0.9 ± 0.1	11 ± 1.6*
OPN-CT	1.6 ± 0.5	9.6 ± 1.9*	2 ± 0.7
NT10k	4.2 ± 1.1**	1.1 ± 0.2	5 ± 2.4**
r OPN (GK)	12.6± 2.1*	10.4 ±1.6*	11 ± 1.7*
r OPN (CKII)	8. ± 1.8*	10.3 ±1.6*	12 ± 3.1*
r OPN (CKI)	10.± 1.9*	9.9 ±2.3*	8 ± 2.6*
r OPN (PKG)	0.8± 0.4	8.7 ±2.0*	1.0 ± 0.1*

Unphosphorylated OPN			
	Haptotactic index	Chemotactic index	Spreading index
control	1 ± 0.3	1 ± 0.2	1± 0.1
OPN	3.6 ± 0.6	11.4 ± 1.8	2 ± 0.7
OPN-NT	0.6 ± 0.2	0.9 ± 0.2	11 ± 1.5
OPN-CT	1.3 ± 0.6	9.6 ± 1.9	2 ± 0.8
NT10k	1.8 ± 0.9	1.1 ± 0.1	7 ± 0.5
r OPN	1.5 ± 0.1	10.5 ± 2.2	2.4 ± 0.3

Absence of the CD44 Gene Prevents Sarcoma Metastasis

Running Title: CD44 in Metastasis

Key Words: CD44, gene-targeted mice, p53, APC, metastases

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Abstract

To test the role of the CD44 gene in tumorigenesis, mice with the min mutation of the APC gene or with the tm1 mutation of the p53 gene were crossed with CD44 knockout mice. The absence of CD44 gene products did not affect tumor incidence or survival, however, mice with disruption of the CD44 gene showed virtually aborted metastasis formation of osteosarcomas in agreement with the role attributed to CD44 variants in cancer spread. Therefore, CD44 gene products are not essential for tumor incidence and growth, but are important in regulating metastasis formation.

Introduction

The transmembrane glycoprotein CD44 is expressed on lymphocytes and macrophages. It serves as a homing receptor that mediates binding to high endothelial venules and has also been implicated in lymphoid development. Lymphocyte activation results in the expression of multiple, alternatively spliced products of the CD44 gene, which are generated by insertion of up to 10 variant exons into the extracellular domain. In pathophysiology, aberrant expression of certain CD44 splice variants has been causally connected to the spread of diverse malignant cells [1,2] and may distinguish metastasizing from non-metastasizing tumors. This function is mediated by the cytokine ligand osteopontin [3]. Additional roles in carcinogenesis have been attributed to various forms of CD44. Expression of this receptor on tumor cells may support tumor growth [4,5], possibly after adhesion to hyaluronate, and signal transduction through CD44 can induce oncogenes such as *ras* [6]. In contrast, the standard form that lacks variant exons may exert suppression of tumor growth and dissemination [7]. The contributions by these diverse CD44 functions to carcinogenesis are not fully elucidated.

Despite ample experimental evidence for a role of some forms of CD44 in malignancy, clinical studies relating expression of CD44 or its variants to prognosis in diverse cancers have remained controversial [8]. Non-conclusive results in patients may have been obtained due to insufficient sensitivity or specificity of the analyses. Thus, determination of mRNA messages for specific CD44 variants expressed by tumors does not detect posttranslational modifications that may affect function [9]. Furthermore, the CD44 receptor expressed on tumors represents one

component of a functional pair with specific ligands that may bind to selective splice variants so that the presence of these ligands contributes to determining the phenotype. Conversely, many conventional rodent models of malignancies are compromised because they rely on the injection of preformed tumor cells, often in non-physiologic locations, rather than on the generation of endogenous tumors. We set out to analyze the role of CD44 in a genetically defined and homogeneous system, which most closely resembles the pathophysiology of human cancers. Mouse models where the role of individual genes is tested by breeding the relevant gene targeted mice with mice that have high susceptibility to tumors due to mutations in tumor suppressor genes have provided substantial insights. We employed two endogenous tumor models using mice with point mutations in tumor suppressor genes with or without targeted deletion of the CD44 gene: Mice with the $APC^{+/min}$ genotype display a predisposition to multiple intestinal neoplasia. The murine Min mutation is a nonsense mutation, which is analogous to mutations found in human autosomal dominantly inherited familial adenomatous polyposis as well as in sporadic colorectal cancers [10]. $APC^{+/min}$ mice develop multiple benign intestinal tumors, whose growth reflects early steps of transformation. Mutations of the p53 gene contribute to the pathogenesis of a large percentage of human cancers. Similarly, mice with one mutant allele of the p53 gene are susceptible to a larger spectrum of tumors, predominantly sarcomas and lymphomas. These mice allow the investigation of malignant dissemination.

Materials and Methods

Mice: Mice with point mutations in tumor suppressor genes, $APC^{+/min}$ bred on C57Bl/6 background or $trp53^{+/tm1}$ on C57Bl/6 background, were obtained from Jackson Laboratory. Either $APC^{+/min}$ mice or $p53^{+/tm1}$ mice were mated with $CD44^{-/-}$ mice that had been back-crossed from 129 to C57BL/6 for 4 generations [10]. The genotypes were assessed using PCR from genomic DNA [10,11,12] and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody IM7 (Pharmingen). Siblings were housed in groups of 1-4 per cage at the Redstone Animal Facility (DFCI) in alternate 12-hr light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered ad libitum and room temperature was kept at 25°C. The colony was frequently tested for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee and the mice were frequently seen by a veterinarian.

Inheritance: Mice with disrupted CD44 gene were mated with heterozygotes for point mutations of the relevant tumor suppressor gene yielding mice that were hemizygous for CD44 and either wild type or heterozygous for the tumor suppressor gene. Those two genotypes were interbred which is expected to result in equal 12.5% representation of the genotypes of interest according to Mendelian inheritance (the remaining 2 x 25% are $CD44^{+/-}$). The litters from this second generation mating were screened. In the p53 related part of the study, 292 mice were analyzed, of which 16 were $trp53^{+/+}CD44^{+/+}$, 36 mice were $trp53^{+/+}CD44^{-/-}$, 24 mice had the genotype $trp53^{+/tm1}CD44^{+/+}$,

and 26 mice had the genotype trp53$^{+/tm1}$CD44$^{-/-}$. In the APC related part of the study, 217 mice were screened with the distribution of APC$^{+/+}$CD44$^{+/+}$ 21 mice, APC$^{+/+}$CD44$^{-/-}$ 20 mice, APC$^{+/min}$CD44$^{+/+}$ 10 mice, and APC$^{+/min}$CD44$^{-/-}$ 15 mice.

Necropsy: The animals were checked at least every 12 hours and total necropsy was performed on mice found moribund. Organs were fixed in formalin and hematoxilin-eosin slides were prepared for histologic analysis. Histologic evaluation was performed by an investigator blinded to the CD44 status of the samples.

Osteosarcoma metastases were observed in livers, lungs and occasionally spleens. Enumeration of osteosarcoma metastases was done on step sections from livers and lungs. For this purpose, every tenth microtome cut corresponding to step thickness of 60 μm was analyzed.

Enumeration of intestinal polyps: Entire intestines from stomach to rectum were extracted, washed in PBS, fixed in 10% buffered formalin, and the number of polyps was counted under a dissection microscope. As controls, intestines from 3 APC$^{+/+}$CD44$^{+/+}$ at the ages of 420-442 days and 3 APC$^{+/+}$CD44$^{-/-}$ mice at the ages of 433-442 days were examined for spontaneous polyps.

Statistical evaluation: The data sets were analyzed for statistically significant differences at 95% confidence by t-test (after confirmation of normal distribution and equal variance) and by Wilcoxon-Mann-Whitney test (after testing for equal distribution).

Results

Absence of CD44 prevents tumor metastasis. Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the CD44 gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One $trp53^{+/tm1}CD44^{-/-}$ mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from $trp53^{+/tm1}$ mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 $CD44^{+/+}$ mice and 1 metastasis in 4 $CD44^{-/-}$ mice (Figure 1A-D). One $CD44^{+/+}$ mouse also displayed a macroscopically visible metastasis in the spleen. All 6 $CD44^{+/+}$ mice had multiple osteosarcoma metastases while in 4 $CD44^{-/-}$ mice only one individual lung metastasis was detected. Consistently, CD44 expression was detected in the osteosarcomas of $CD44^{+/+}$ mice (Figure 2).

Absence of CD44 does not alter the phenotype of benign tumors. The intestinal polyps caused by mutation in the APC gene grow non-invasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia [15]. Histology was performed on the largest intestinal polyps from each $APC^{+/min}$ mouse to assess malignancy. Consistent with previous reports, these tumors are non-invasive as judged by intact basement membranes in all cases (Figure 1G-H). No metastases were observed in other organs (3 histologic sections per organ). These results were not affected by the presence or absence of CD44. Histologic findings included ectopic hepatic hematopoiesis and bone marrow siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps.

CD44 does not affect tumor incidence. We tested whether deletion of the CD44 gene alters tumor incidence as judged by the number of intestinal polyps in mice with one mutated APC allele. All $APC^{+/min}$ mice succumbed to intestinal polyposis. At the time of death, $APC^{+/min}$ mice had developed around a mean of 66 polyps in $CD44^{+/+}$ background and 58 polyps in $CD44^{-/-}$ background (Figure 3A) suggesting that the tumor development in this model does not depend on the presence of CD44 gene products.

Mice with the $trp53^{+/tm1}$ genotype developed predominantly sarcomas and lymphomas. The mesenchymal tumors were diagnosed as fibrosarcomas, osteosarcomas, hemangiosarcomas, and histiocytic sarcomas. Their incidence, associated life span, and tumor weight upon death were not affected by the presence or absence of the CD44 gene (Figure 3B). Like the osteosarcomas, the fibrosarcomas were mostly located on the lower back. Sporadic carcinomas also occurred in $trp53^{+/tm1}$ mice independently of their CD44 status with one case of squamous cell carcinoma in a $trp53^{+/tm1}CD44^{+/+}$ mouse and one incident of lung carcinoma among the $trp53^{+/tm1}CD44^{-/-}$ mice (Figure 3C).

There were 4 cases (17%) of lymphoma, typical of those observed in $p53^{+/tm1}$ mice [12,13], in $trp53^{+/tm1}CD44^{+/+}$ mice with an associated mean life span of 445 days compared to 6 cases (23%) of lymphomas, resembling anaplastic large cell lymphomas [14], in $trp53^{+/tm1}CD44^{-/-}$ mice with an associated life span of 503 days (data not shown). The morphology of the lymphoid malignancies in $CD44^{-/-}$ mice appeared unusual, but requires further characterization.

Several mice with one mutant p53 allele had multiple tumors. In trp53$^{+/tm1}$CD44$^{+/+}$ mice, one osteosarcoma occurred together with histiocytic sarcoma. Frequently, lymphomas were diagnosed in conjunction with solid tumors. One trp53$^{+/tm1}$CD44$^{+/+}$ mouse had lymphoma and osteosarcoma. In trp53$^{+/tm1}$CD44$^{-/-}$ mice, lymphoma was seen in conjunction with osteosarcoma, fibrosarcoma, and histiocytic sarcoma in one case each.

CD44 does not affect survival. The mice with one mutant allele of the APC gene developed symptoms of ruffled fur, bloated abdomen, black stools followed by lethargy and succumbed around a mean of 209 days (range 133-350 days) of age for CD44$^{+/+}$ background or 236 days (range 96-326 days) of age for CD44$^{-/-}$ background (Figure 4A). At the end of their life span, two of the CD44$^{-/-}$ mice also suffered from rectal prolapse. None of the APC$^{+/+}$ mice died during the 420 days period of observation, regardless of their CD44 status.

Mice with one mutant p53 allele developed various tumors with a predominance of sarcomas and lymphomas and had a 50% survival of around 470 days of age regardless of their CD44 genotype (Figure 4B). The period of observation was limited to 600 days, at which point 1 of 24 trp53$^{+/tm1}$CD44$^{+/+}$ mice (4%) was still alive and 6 of 26 trp53$^{+/tm1}$CD44$^{-/-}$ mice (23%) survived. In the control groups 14 of 16 trp53$^{+/+}$CD44$^{+/+}$ mice (87%) and 30 of 36 trp53$^{+/+}$CD44$^{-/-}$ mice (83%) were alive. Conversely, 1 trp53$^{+/tm1}$CD44$^{+/+}$ mouse (4%), 3 trp53$^{+/tm1}$CD44$^{-/-}$ mice (11%), 1 trp53$^{+/+}$CD44$^{+/+}$ mouse (6%), and 5 trp53$^{+/+}$CD44$^{-/-}$ mice (14%) died without detectable signs of malignancies. The higher incidence of deaths unrelated to neoplasms (8 CD44$^{-/-}$ mice of 50 when

disregarding the trp53 status, compared to 2 $CD44^{+/+}$ mice of 40) implies that the lack of the CD44 gene may increase the susceptibility to other pathogenic influences.

Discussion

Diverse roles in cancer have been ascribed to various CD44 gene products, but their contributions to endogenous tumors have not been studied. Here, we have tested the consequences of targeted deletion of the CD44 gene in endogenous tumors caused by mutations in two distinct tumor suppressor genes, which are also frequently mutated in human cancers. We show that the absence of CD44 gene products virtually abrogates osteosarcoma metastasis. In contrast, we did not find evidence for a role of CD44 in tumor growth and survival.

In various cancers, expression of CD44 splice variants is necessary and sufficient to cause metastasis formation [1,2]. In the present study, the role of CD44 in malignancy of non-hematopoietic origin was limited to inducing dissemination (Figure 1), corroborating the role of CD44 as a metastasis gene in solid tumors. The observation that the expression of CD44 is sufficient to confer metastatic properties to primary osteosarcoma cells, but that the expression of multiple CD44 variants on intestinal adenomatous polyps [15] is not associated with invasive growth, may be accounted for by dominance of metastasis suppressor gene products over gene products that induce dissemination [24]. Intestinal cells frequently express the adhesion molecule DCC which may prevent CD44 mediated invasion. Alternatively, metastasis formation by the intestinal polyps may be suppressed by elevated β-catenin secondary to loss of APC protein function. This decreases enterocyte crypt villus migration [25] and may prevent invasive behavior. Osteosarcoma cells, in contrast, do not display prominent expression of metastasis suppressor genes so that CD44 is sufficient to cause a malignant phenotype.

Various genetic influences can affect tumor multiplicity in $APC^{+/min}$ mice. They include genes for cell cycle control, DNA repair, and metalloproteinases. The genetic modifier Mom1 encodes a secretory phospholipase, Pla2g2a, expressed throughout the intestinal tract. The active allele of Pla2g2a leads to a reduction in the growth rate and multiplicity of intestinal adenomas [26]. $APC^{+/min}$ mice homozygous for a null allele of p53 developed significantly more intestinal adenomas than those homozygous for the wild-type allele of p53. Similarly, the intact DNA mismatch repair gene Pms2 reduces the number of intestinal tumors as compared to mice with targeted deletion of this gene [27]. In contrast, deletion of the gene for the metalloproteinase matrilysin leads to substantial reduction in intestinal tumors, despite a lack of destruction of the basement membrane by these polyps [28]. The intestinal polyps caused by the APC gene mutation express various splice variants of CD44 at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia [15], however, the contributions by CD44 gene products to the pathogenesis of the intestinal polyps was unknown. In this study, numbers of polyps and associated life spans were not influenced by the absence of CD44 gene products. Subjectively, the size of the individual polyps also did not appear to be compromised.

One interpretation for the lack of an effect on tumor incidence and survival by the $CD44^{-/-}$ genotype might be that a polymorphic modifier, linked to the CD44 locus, segregates with it and supersedes the influence of CD44 on the development of intestinal adenomas. The tumor susceptibility locus Scc1 might be a candidate [29]. This is unlikely, because quantitative trait

loci, including Scc-1, depend strongly on interlocus interactions for influencing tumorigenesis [30] so that co-segregation of one modifier could not influence the phenotype. It is generally improbable that genomic heterogeneity would account for this possibility, because 97% of the genome are derived form the C57Bl/6 strain.

Expression of CD44 on tumors has been described to not only affect metastatic spread but also tumor growth [4,5] and induction of oncogenes such as *ras* [10]. This opened the possibility that deletion of the CD44 gene might influence disease progression. In $APC^{+/min}$ mice, incidence of polyps and associated life spans were, however, not altered. Similarly, incidence, survival, and tumor weight of sarcomas in $trp53^{+/tm1}$ mice were not influenced by the absence of CD44, arguing against a prominent role for CD44 in early transformation or tumor growth. In contrast, the dissemination of osteosarcomas was virtually abrogated by the absence of CD44 gene products (29 microscopically and macroscopically identified metastases in 6 $CD44^{+/+}$ mice, compared to 1 metastasis identified in 4 $CD44^{-/-}$ mice). We have identified metastasis genes as stress response genes, which constitute a unique group of cancer-related biomolecules. They are dysregulated in cancer at the levels of expression or splicing [24]. The present results confirm the role of CD44 as a metastasis gene and refine our insights into the contributions of CD44 to cancer.

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Legends to Figures and Tables

Figure 1: Tumor invasiveness. A-F) Osteosarcomas in trp53$^{+/tm1}$ mice and their metastases in lungs and liver. A) primary tumor in CD44$^{+/+}$ background B) representative lung metastasis in CD44$^{+/+}$ background C) representative liver metastasis in CD44$^{+/+}$ background E) primary tumor in CD44$^{-/-}$ background F) only metastasis detected in CD44$^{-/-}$ background (hematoxilin-eosin). F) Incidence of metastases in livers and lungs from osteosarcoma bearing mice. G-H) Histology of representative intestinal polyps from APC$^{+/min}$ mice. The largest polyp from each intestine was sectioned in the middle and stained with hematoxilin-eosin for histologic assessment of signs for malignancy. Regardless of the presence of the CD44 gene, the basement membrane remains intact (arrows), G) CD44$^{+/+}$, H) CD44$^{-/-}$.

Figure 2: Expression of CD44. A) PCR for genotyping. B) Expression of CD44 by flow cytometry. C) Immunohistochemistry for CD44 expression in osteosarcomas.

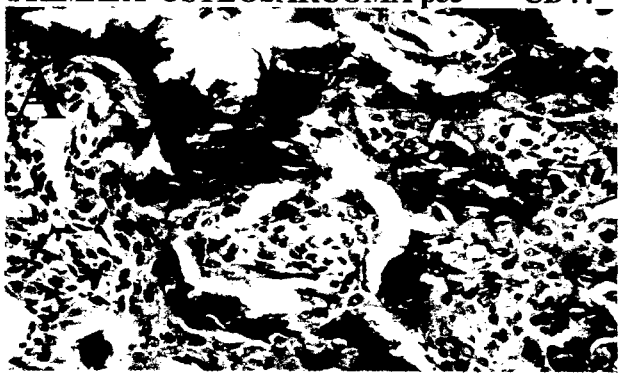
Figure 3: Tumor incidence. A) The numbers of intestinal polyps were counted in APC$^{+/min}$ mice at the end of their life span (average 209 days for 10 APC$^{+/min}$CD44$^{+/+}$ and 236 days for 15 APC$^{+/min}$CD44$^{-/-}$). The incidence of spontaneous polyps was assessed at an average of 433 days for 3 APC$^{+/+}$CD44$^{+/+}$ and 437 days for 3 APC$^{+/+}$CD44$^{-/-}$ mice. Symbols indicate individual data points; mean values are presented as horizontal lines. B) Incidence of sarcomas in trp53$^{+/tm1}$ mice. Independent of the CD44 gene, mice are susceptible to sarcomas of various tissue specificity. C57BL/6 mice with wild-type p53 gene also develop spontaneous sarcomas at low frequency. Life

span and tumor weight are indicated as mean ± standard error. C) Histology of sporadic carcinomas in trp53$^{+/tm1}$ mice including a squamous cell carcinoma (CD44$^{+/+}$) (left) and a lung carcinoma (CD44$^{-/-}$) (right). The slides are stained with hematoxilin-eosin. f = female, m = male, $p>0.05$ = not significantly different with a probability of error of 5%.

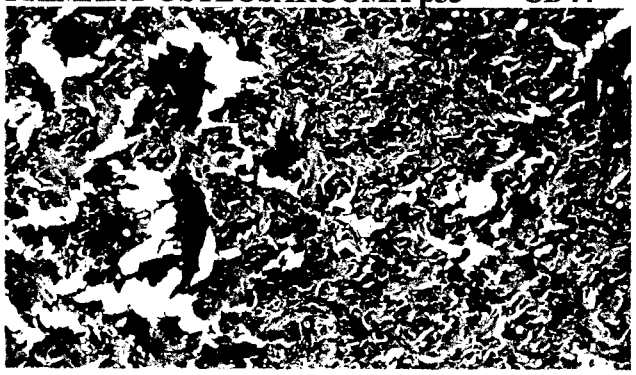
Figure 4: Kaplan-Meier survival curves. A) Survival of APC$^{+/min}$ mice with wild-type (filled squares) or deleted (filled circles) CD44 gene. All APC$^{+/+}$ mice survived the 420 days period of observation. B) Survival of trp53$^{+/tm1}$ mice. Within 600 days, mice with the trp53$^{+/tm1}$ genotype succumbed to various tumors independently of their CD44 status leading to survival of 4% or 1 mouse (CD44$^{+/+}$, open triangles) and 23% or 6 mice (CD44$^{-/-}$, closed squares). Control mice with wild type trp53 gene had survival rates of 87% or 14 mice (CD44$^{+/+}$, closed triangles) and 83% or 30 mice (CD44$^{-/-}$, open squares).

Figure 1

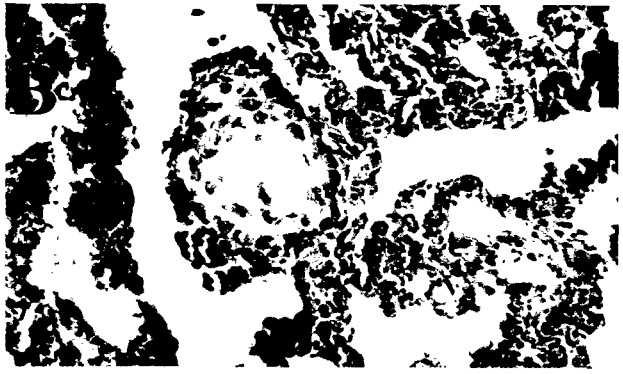
PRIMARY OSTEOSARCOMA $p53^{+/trp1}$ $CD44^{+/+}$



PRIMARY OSTEOSARCOMA $p53^{+/trp1}$ $CD44^{-/-}$

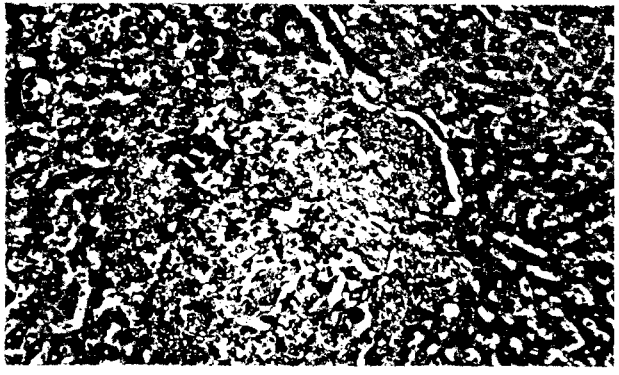


LUNG METASTASIS $p53^{+/trp1}$ $CD44^{+/+}$



LUNG METASTASIS $p53^{+/trp1}$ $CD44^{-/-}$

LIVER METASTASIS $p53^{+/trp1}$ $CD44^{+/+}$



F

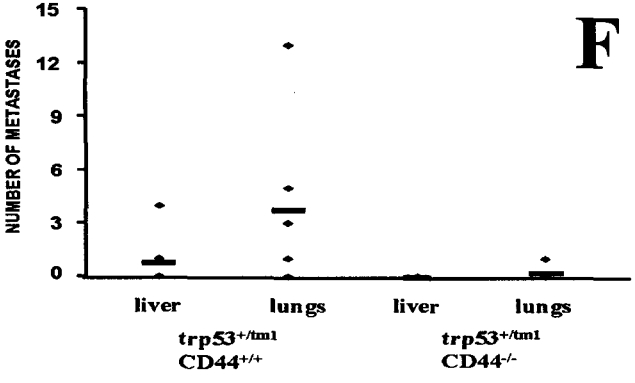


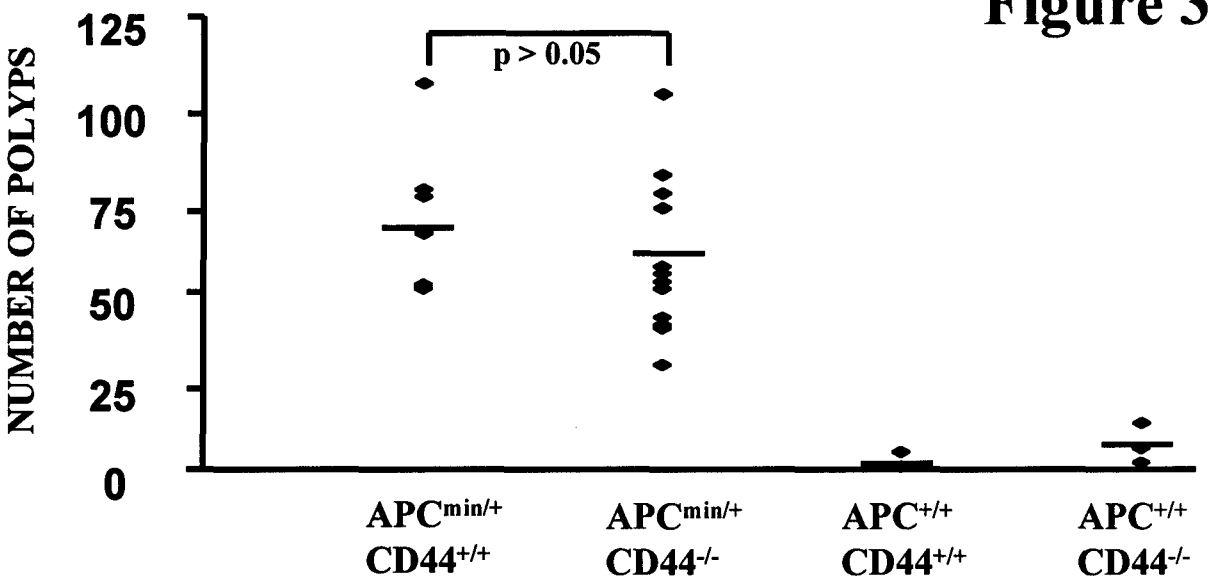
Figure 1

$APC^{+/min}$ $CD44^{+/+}$

$APC^{+/min}$ $CD44^{-/-}$

Figure 3

A

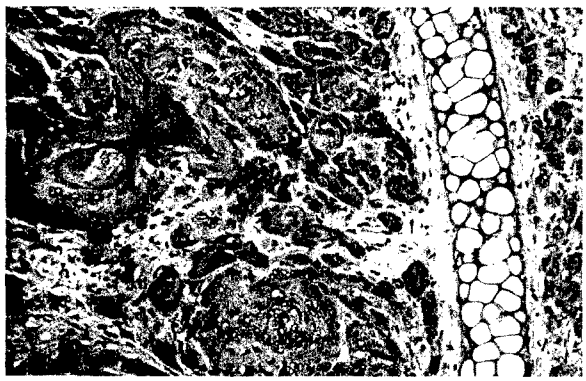


B

TUMOR	INCIDENCE	LIFE SPAN	WEIGHT
osteosarcoma			
$trp53^{tm1/+}CD44^{+/}$	6 (25%) 5 f, 1 m	532 ± 30 d] p > 0.05	7.7 ± 2.4 g] p > 0.05
$trp53^{tm1/+}CD44^{-/-}$	4 (15%) 3 f, 1 m	467 ± 56 d]	6.1 ± 1.7 g]
fibrosarcoma			
$trp53^{tm1/+}CD44^{+/+}$	7 (29%) 4 f, 3 m	410 ± 23 d] p > 0.05	21.9 ± 5.0 g] p > 0.05
$trp53^{tm1/+}CD44^{-/-}$	7 (27%) 5 f, 2 m	403 ± 30 d]	12.3 ± 2.9 g]
hemangiosarcoma			
$trp53^{tm1/+}CD44^{+/+}$	3 (12%) 2 f, 1 m	304 ± 51 d	10.7 ± 8.1 g
$trp53^{tm1/+}CD44^{-/-}$	0 (0%)		
$trp53^{+/+}$ $CD44^{-/-}$	1 (3%) 1 m	595 d	2.9 g
histiocytic sarcoma			
$trp53^{tm1/+}CD44^{+/+}$	2 (8%) 2 m	589/590 d	
$trp53^{tm1/+}CD44^{-/-}$	1 (4%) 1 f	420 d	
$trp53^{+/+}$ $CD44^{+/+}$	1 (6%) 1 m	600 d	

C

SQUAMOUS CELL CARCINOMA ($trp53^{+/tm1}$ $CD44^{+/+}$)



LUNG CARCINOMA ($trp53^{+/tm1}$ $CD44^{-/-}$)

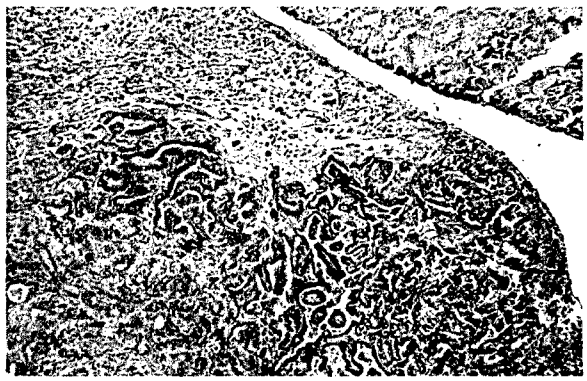
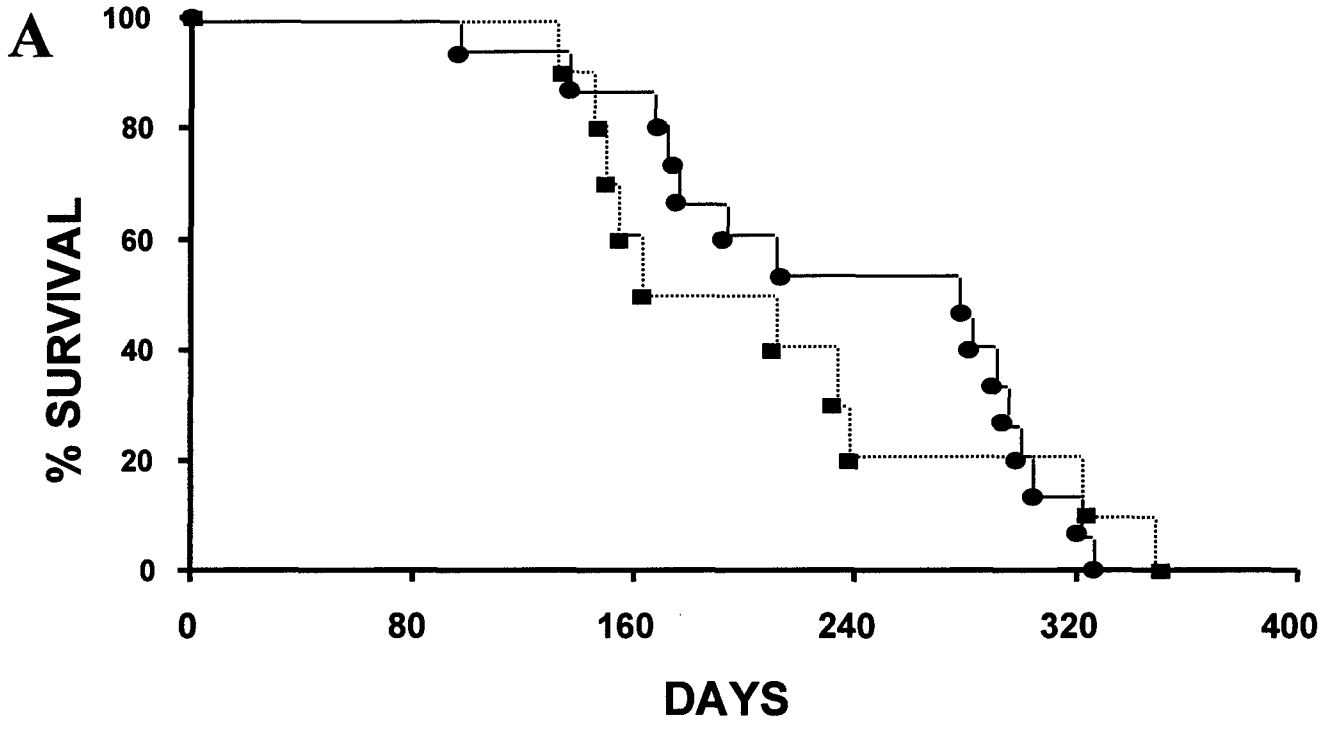
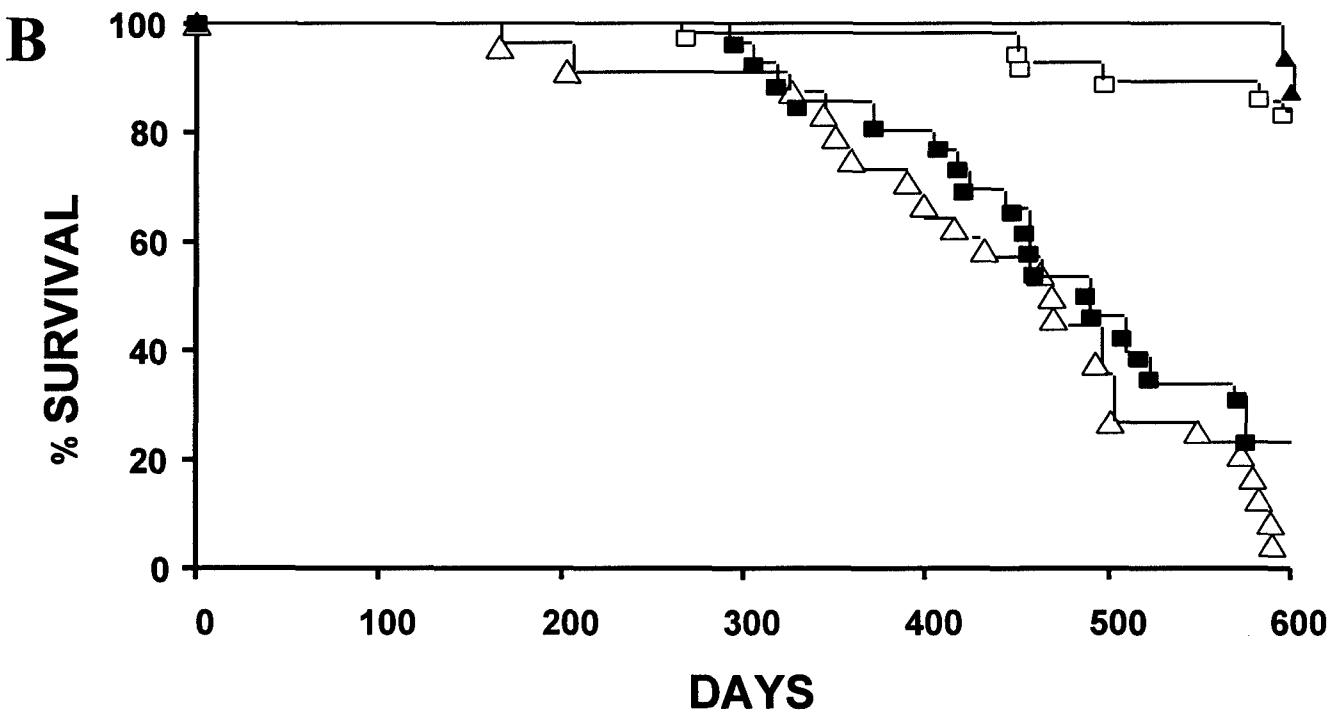


Figure 4


A
100
80
60
40
20
0
% SURVIVAL
0
80
160
240
320
400
DAYS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Molecular mechanisms of tumor dissemination in primary and metastatic brain cancers

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ABSTRACT: Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. Tumor dissemination distinguishes malignant from benign neoplasms and is mediated by homing receptors, their ligands, and proteinases. The homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases. Its engagement by osteopontin physiologically induces macrophage chemotaxis, a mechanism that may be utilized by metastatic brain tumors in the process of dissemination. In host defense, osteopontin and its receptors, CD44 and integrin $\alpha_v\beta_3$, play key roles in mediating delayed type hypersensitivity responses by activating macrophages to induce Th1 cytokines while inhibiting Th2 cytokines. Other metastasis associated gene products similarly contribute to host defenses. Hence, cancer spread is regulated by a set of developmentally nonessential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization. Function of the relevant gene products is extensively modified post-transcriptionally and their dysregulation in cancer occurs on the levels of expression and splicing. Consistent patterns of organ preference by malignancies of particular tissue origin suggest a necessary connection between loss of growth control and senescence genes and expression of genes mediating the dissemination of tumor cells. © 2000 Elsevier Science Inc.

KEY WORDS: Invasion, Homing receptors, Cytokines, Proteinases, Stress response.

MOLECULAR CHARACTERISTICS OF CANCER

The most prominent feature of malignancy is dysregulated cell cycle progression. Division of cancer cells leads to formation of more cancer cells indicating that the characteristics of transformation originate in genetic changes. The underlying defects causing uncontrolled proliferation are gain of function mutations in oncogenes or loss of function mutations in tumor suppressor genes. However, most somatic cells, with few exceptions such as stem cells, die after a finite number of cell divisions, a phenomenon described as senescence. Replicative senescence begins after fertilization and is genetically dominantly controlled. For cancer to occur, there must be a loss of function in senescence genes or a gain of function in telomerase to give rise to a largely unlimited number of cell divisions. Finally, cancer is distinguished from benign tumors by its faculty to generate metastases. In contrast to earlier models, metastasis formation is a process of active cell migration and invasion rather than the passive dyslocation of cells in the blood or lymph flow. Whether a neoplasm metastasizes and to which target organs is determined by motility associated molecules expressed by the tumor cells.

INVASIVENESS OF BRAIN TUMORS

The brain is unique as a target organ for metastatic growth because it is surrounded by the blood—brain barrier and it lacks lymphatic drainage. Nevertheless, certain malignancies display a preference for dissemination to the central nervous system (CNS). Brain metastases from colon and breast cancers are often single, whereas melanoma and lung cancer have a greater tendency to produce multiple colonies. At autopsy, up to 80% of melanoma patients have CNS lesions [20]. Invasion of brain cancer cells typically proceeds along anatomic structures that are rich in extracellular matrix proteins, including basement membranes of blood vessels and the glial limitans externa [4] and has been attributed to specific motility-associated receptors, their ligands and proteinases [6] (Table 1). Specifically, the homing receptor CD44 is frequently expressed on primary brain tumors and brain metastases [10,12,15]. Its ligand osteopontin has also been described to be secreted by malignant gliomas [5,16,22].

THE PHYSIOLOGIC ROLES OF METASTASIS GENES

To understand the process of metastasis formation we have studied the physiologic importance of the relevant gene products. We have investigated the cytokine osteopontin and its receptor CD44 [25–27]. The engagement of CD44 by osteopontin induces macrophage chemotaxis, a process that may be utilized by metastatic brain tumors in the process of dissemination [26]. Gene-targeted mice deficient in osteopontin or CD44 are fertile and developmentally normal, a trait that is shared by other knockouts for genes that are believed to be important in metastatic spread. Several observations implied that osteopontin may act as a stress

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TABLE 1

METASTASIS-MEDIATING MOLECULES IN BRAIN TUMORS

Tumor	Cytokines	Receptors	Proteinases
Primary brain tumors			
Glioblastoma	Urokinase plasminogen activator, interleukin-8, osteopontin	CD44	Gelatinase-B, active Gelatinase-A, Cathepsin L
Astrocytoma	Hepatocyte growth factor/scatter factor, interleukin-8	c-Met	MT1-MMP, MT2-MMP
Medulloblastoma		polysialylated NCAM	
Metastatic brain tumors			
Melanoma		Neurotrophin receptor	Heparanase
Lung cancer	Urokinase plasminogen activator		
Breast cancer	Urokinase plasminogen activator	Interleukin-6 receptor, CD44	
Prostate cancer		Insulin-like growth factor receptor	
Renal cancer		Interleukin-6 receptor	

Specific receptors, ligands (migration inducing cytokines), and proteinases have been associated with the invasive behavior of individual primary and metastatic brain tumors. MMP, matrix metalloproteinase.

response gene: (1) the osteopontin promoter contains an acute phase responsive element [9] and a phorbol ester responsive element to which the redox sensitive transcription factors Jun and Fos may bind [13], (2) osteopontin expression by T-lymphocytes, macrophages, and osteoclasts does not occur at rest but is activation dependent and is associated with host resistance [13], (3) osteopontin exerts anti-oxidant effects and prevents cell damage in response to a large number of noxious influences [23], (4) the osteopontin gene knockout results in defective wound healing [11]. In host defense, CD44 and its ligand osteopontin play a key role in mediating delayed type hypersensitivity responses by skewing the pattern of cytokines secreted from macrophages to favor the in-

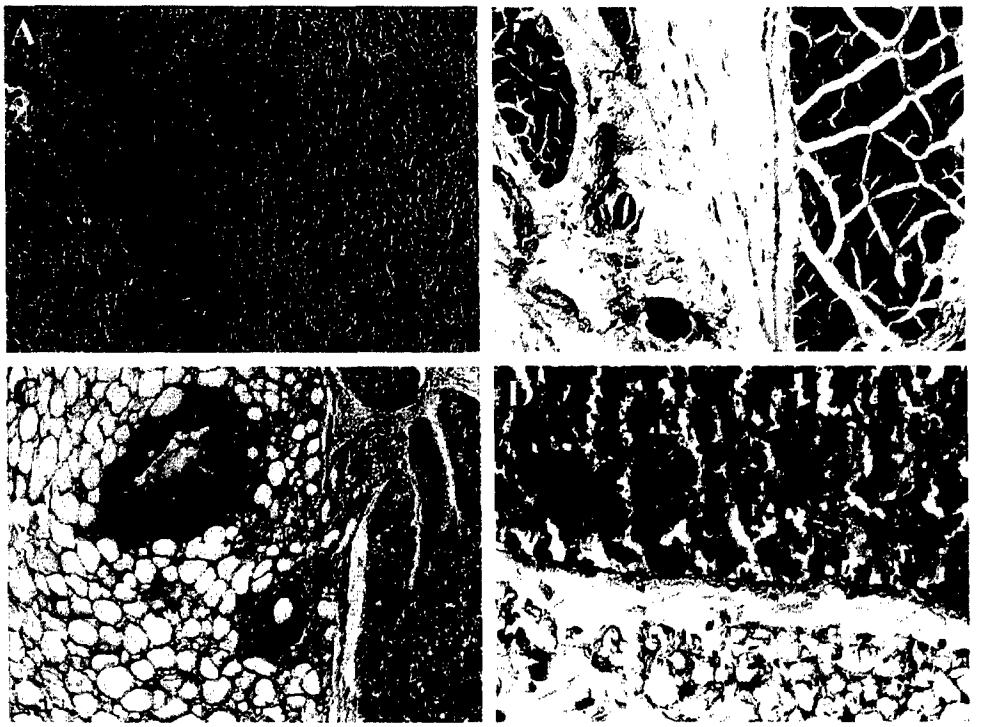

FIG. 1. Absence of a delayed type hypersensitivity response in mice lacking the osteopontin gene. 250 μg polyvinyl pyrrolidone (PVP) in 500 μl phosphate-buffered saline (PBS) was injected subcutaneously into the hind limb of C57BL/6 wildtype mice or gene targeted C57BL/6 OPN$^{-/-}$ mice which do not express the osteopontin gene product. After 5 days, histologic analysis of the injection site was performed; 5-μm serial sections were stained with hematoxilin-eosin stain. (A) Injection of PVP into C57BL/6; (B) injection of PBS (vehicle control) into C57BL/6; (C) injection of PVP into C57BL/6 OPN$^{-/-}$ and (D) injection of PVP in conjunction with 10 μg purified osteopontin into C57BL/6 OPN$^{-/-}$. Original magnification 200×.

TABLE 2

GENES ASSOCIATED WITH MALIGNANCIES

Genes	Function	Examples
Oncogenes	Growth factors	EGF, PDGF
	Growth factor receptors	HER-2, erb-B
	Signal transduction molecules associated with growth factor receptors	Akt, Abl, Ras
Tumor suppressor genes	Receptors	DCC,PTC
	Signal transduction molecules	p53, Rb,APC
Senescence genes	Cell cycle regulators	p53,Rb,p21,Fos
Senescence suppressor genes	Regulators of telomere length	Telomerase
Metastasis genes	Homing receptors and their ligands	CD44, selectins, osteopontin
	Proteinases	MMPs
Metastasis suppressor genes	Adhesion receptors	cadherins, L-CAM, KAI1
	Proteinase inhibitors	TIMPs
Mutator genes	Mismatch repair	MSH, PMS
	Base excission repair	Uracil DNA glycosylase
	Nucleotide excission repair	ERCC
	Repair of double strand breaks	XRCC,RAD50,NSB1

The classical cancer genes (oncogones and tumor suppressor genes) control cell replication. For cancer to occur, additional functions need to be dysregulated: genes that cause cellular senescence have to be inactivated and expression of gene products that mediate metastasis formation is essential. For cell cycle progression and cell dissemination alike, there is a physiologic balance that may be disturbed by excessive activity of promoters or by diminished function of suppressors. Defects in mutator genes give rise to alterations in other cancer-associated genes putting mutator genes into the position of predisposing factors rather than direct contributors to the malignant phenotype.

duction of cellular immunity and to suppress humoral immunity. The interaction of osteopontin with its integrin receptor $a_V\beta_3$ on macrophages stimulates the production of Th1 cytokines while engagement of CD44 by osteopontin concomitantly inhibits the secretion of Th2 cytokines [2]. A classical model of delayed type hypersensitivity is granuloma formation. Foreign body granulomas can be induced by subcutaneous injection of polyvinyl pyrrolidone. After 5 days, control mice display pronounced influx of macrophages and a strong local immune response. In contrast, mice lacking the osteopontin gene due to targeted mutation barely show any immunological reaction to the injection (Fig. 1). In contrast, mice lacking the CD44 gene display excessive granuloma formation following challenge [17] which may reflect combined Th1 and Th2 immunity after engagement of integrin receptors by osteopontin in the absence of ligation of CD44.

Preliminary experiments have suggested that $CD4^+$ T-cells secrete the osteopontin that induces macrophages to selectively promote delayed type immune responses. The observation that macrophages may themselves produce osteopontin after stimulation with lipopolysaccharide raises questions regarding its potential relevance to this process. Macrophage-derived osteopontin is competent for inducing chemotaxis but not delayed type hypersensitivity which may reflect structural differences from the T-cell secreted molecule. In fact, macrophage osteopontin has lost part of its sequence by alternative splicing [Ashkar and Weber, unpublished observations] which could lead to efficient engagement of CD44 with ensuing chemotaxis but to impaired ligation of integrin receptors. Malignant cells often secrete a form of osteopontin that resembles the macrophage-derived protein in that it may be hypophosphorylated or a splice variant that has a deletion in its N-terminal (integrin binding) portion [8] and this molecule may contribute to metastatic spread [25] by inducing tumor cell migration. Concomitantly, tumor-derived modified osteopontin may ligate CD44 on macrophages without engagement of its integrin receptors [19]. This leads to suppression of Th2 cytokines while Th1 cytokines cannot be efficiently secreted since other physiologic inducers of Th1 cytokines are substantially less potent. This form of osteopontin action may represent a mechanism of immune evasion.

Tumor dissemination depends on neovascularization. Physiologically, blood vessel formation may be initiated in two settings. The modeling of the cardiovascular system is largely restricted to early development, while in the healthy adult organism, angiogenesis is a rare occurrence that arises predominantly in healing after tissue damage. Morphogenic and stress induced blood vessel generation are mediated by distinct sets of genes. Several pieces of evidence imply a role for osteopontin and its receptors in the latter form of neovascularization. A splice variant of CD44 is involved in endothelial cell proliferation, migration, and angiogenesis [7, 21]. The integrin $a_V\beta_3$ is of particular importance in angiogenesis due to its selective expression on growing blood vessels. Antagonists of integrin $a_V\beta_3$ promote tumor regression by inhibiting neovascularization [1,3] and angiogenesis induced by bFGF or by TNFα is also inhibitable by a monoclonal antibody to the integrin $a_V\beta_3$. Coordinate expression of β_3-integrins and osteopontin by regenerating endothelial cells [11] and during in vitro blood vessel formation [14] stimulates migration through cooperative mechanisms involving activation of integrin $a_V\beta_3$ ligation by thrombin cleavage of osteopontin [18].

CONCLUSIONS

We conclude, based on our own observations in conjunction with data from the literature, that the topology of cancer spread is regulated by a set of developmentally non-essential genes which physiologically mediate stress responses, inflammation, wound healing, and neovascularization and are normally expressed by activated lymphocytes and macrophages [24]. Function of the relevant gene products is extensively modified post-transcriptionally which allows for quick activation in stress situations and may encode organ specificity. This code for targets in the homing process may cause dissemination to distant organs, such as brain metastases in melanoma or lung cancer, or it may lead to locally invasive growth as is the case in malignant glioma or in chondrosarcoma. In both scenarios, locally destructive growth by malignant glioma and brain metastases from distant primary tumors, the mechanism of invasion is determined by engagement of molecules that are physiologically used by macrophages and lymphocytes to enter the central nervous system in the context of host defenses, including infection, inflammation, or ischemia.

Indicative of basic mechanisms of homeostasis in human biology, all groups of genes involved in malignancies consist of promoting and suppressing components. Loss of function in one group or gain of function in the counterbalancing group may each affect the balance of forces and constitute a predisposing factor for malignant growth. Thus, mutations that enhance the function of oncogenes and mutations that inhibit the function of tumor suppressor genes equally pose a risk for uncontrolled growth of the affected cells and similar relationships hold for senescence genes and metastasis genes and their respective suppressors (Table 2). Furthermore, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection among dysregulated cell cycle control genes (gain of function of oncogenes or loss of function of tumor suppressor genes), suppression of senescence genes, and expression of genes mediating the dissemination of tumor cells. Today, the molecular basis for this connection is largely unknown.

ACKNOWLEDGEMENTS

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HYPOTHESIS

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Stress response genes: the genes that make cancer metastasize

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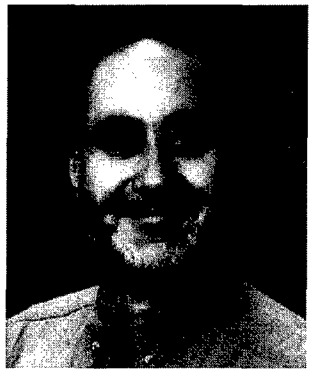

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Abstract Cancer is characterized by dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The topology of cancer spread is mediated by a set of developmentally nonessential genes which are physiologically involved in stress responses, inflammation, wound healing, and neovascularization. The function of these gene products is extensively modified posttranscriptionally. In cancer, metastasis genes are dysregulated at the levels of expression or splicing. These genes constitute a unique group of cancer-related biomolecules.

Key words Cancer · Metastasis · Stress response · Posttranslational modification · Knockout mouse

Introduction

What are the traits that make a killer? This question has intrigued not only fans of detective stories but is also most prominent in the minds of cancer researchers. Here the characteristics of the killer are dysregulated growth control, overcoming of replicative senescence, and metastasis formation. The division of normal cells is tightly controlled by dependence on checkpoints, which are pauses during the cell cycle in which the fidelity of DNA replication and chromosome segregation are monitored. It is regulated by proto-oncogenes, incorporating genes for growth factors, their receptors, and associated intracellular signal transduction molecules. Antagonistic to oncogenes are tumor suppressor genes which normally provide the brakes on cell proliferation. In contrast, cancer is independent of these control mechanisms. Even with defective growth control, however, a cell could never form a tumor of substantial size because, unless it were a germline cell, it would be subject to replicative senescence, an aging process that proceeds with the number of cell divisions and in extreme cases may lead to a state of crisis. A unique role in overcoming replicative senescence is played by the enzyme telomerase, which is expressed in virtually all tumor cells but is ab-

sent from most normal cells. It prevents telomere shortening with increased number of cell divisions, which would eventually cause genomic instability. Mutator genes which encode DNA repair enzymes might be more accurately referred to as meta-oncogenes because their defects give rise to mutations in oncogenes and tumor suppressor genes. Finally, most cells, with the exception of blood and immune cells, grow anchored in their microenvironment whereas cancer cells of particular tissue origin metastasize to specific target organs. The ability of cancer to disseminate throughout the body also sets it apart from benign tumors. However, the classical cancer genes conspicuously do not account for metastasis formation, and current paradigms of cancer have not yet incorporated metastasis genes as a unique group of genes that contributes to the malignant phenotype.

The gene products of stress responses mediate metastasis formation: lessons from knockout mice

The topology of metastasis formation is mediated by the potpourri of homing receptors on the tumor cell surface (Fig. 1) and their ligands and is widely believed to have its physiological correlate in morphogenesis during embryonic development. This would imply that the deficiency of individual metastasis genes should cause defective formation of the relevant target organ. Unexpectedly, knockout mice in which individual genes known to participate in tumor spread were disrupted proved to be fertile and developmentally normal (Table 1). This raises the question: What is the physiological process that goes astray in cancer dissemination?

Despite their diversity, metastasis-associated gene products have several features in common. They comprise a set of genes which physiologically mediate stress responses, including inflammation, wound healing, and neovascularization. Consistently the defects observed in the relevant gene targeted mice are impairments in these areas. This insight resolves some of the paradoxes of metastasis research. In contrast to morphogenesis, invasiveness and tissue damage are in keeping with the normal functions of host defenses that are executed by macrophages and lymphocytes in stress situations. Homing to and expansion in the lymphoid system, typically the first target in metastatic spread, corroborate the notion that cancer metastasis is based on mechanisms normally employed by immunocytes [1]. Differentiation of immune cells proceeds in the context of their tissue of residence, and lymphocytes from Peyer's patches are therefore distinct from cutaneous lymphocytes, and Kupffer cells are distinct from alveolar macrophages. Recognition of topology is encoded in the surface molecules of immune cells, and organ preference by cancer may be derived from this principle.

Table 1 Genes that mediate cancer spread are developmentally nonessential. Cancer dissemination is induced by a group of homing receptors, their ligands, and proteinases in conjunction with their associated signal transduction molecules. These gene products do not play a critical role in organ development or fertility but are necessary for stress responses. Knockout mice have been generated for multiple metastasis associated genes and uniformly show these characteristics. Various integrins have also been linked to metastasis formation but most integrin gene knockouts display developmental defects. This may be due to the loss of multiple receptors after deletion of individual integrin genes. Furthermore, some intergin gene products serve dual roles in stress responses and development (*DTH* delayed type hypersensitivity, *MMP* matrix metalloproteinase, *uPAR* receptor for urokinase-type plasminogen activator)

Gene	Types of cancer	Knockout mouse
Receptors		
uPAR	Prostate cancer, breast cancer [24], gastric carcinoma [25], brain tumors [26]	Defect in leukocyte recruitment and adhesion [8]
CD44	Lymphomas [27], sarcomas [28], colon cancer [29], breast cancer [30]	Excessive granuloma formation [9]
L-selectin	Lymphoma [31]	No DTH to cutaneous antigens [10]
LFA-1	lymphoma [32]	Impaired immune response to alloantigens [11]
ICAM-1	Melanoma [33], lymphoma [34], liver carcinoma [35]	Granulocytosis, diminished DTH, impaired neutrophil homing [12, 13]
IAP (CD47)	Ovarian cancer [36]	Impaired granulocyte activation [14]
Ligands		
Osteopontin	Breast cancer [37], osteosarcoma [38]	Defective wound healing, absence of DTH [15, 16]
Thrombospondin-1	Breast cancer [39], pancreas cancer [40]	Susceptibility to pneumonia [17]
sE-selectin	Gastric cancer [41], breast cancer [42], head and neck cancer [43]	reduced stable adhesion of Leukocytes in inflamed microvasculature [18, 19]
P-selectin	Breast cancer [44], colon cancer [45]	Impaired recruitment of immune cells [20]
Proteinases		
Stromelysin-3 (MMP-3)	Breast cancer [46]	Impaired wound healing [21]
Matrilysin (MMP-7)	Colon cancer [47]	Decreased antimicrobial activity, defective reepithelialization in wounded trachea [22, 23]
Macrophage elastase (MMP-12)	Glioma [48]	Impaired macrophage recruitment [21]

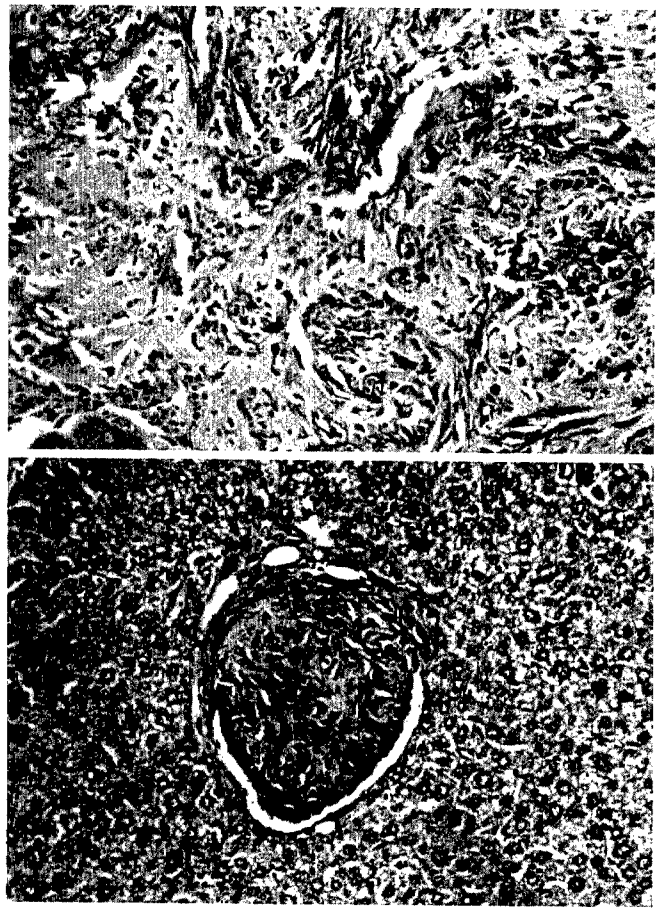

Fig. 1A,B CD44 is essential for metastasis formation by osteosarcoma. C57BL/6 mice with the tm1 point mutation of the *p53* gene are susceptible to osteosarcomas which disseminate to liver and lungs. Shown here are a primary tumor (**A**) and a liver metastasis (**B**). After disruption of both alleles of the CD44 gene metastatic spread is almost completely abrogated while incidence and growth rate of osteosarcomas are unaffected (Weber et al., manuscript in preparation)

The biological activity of metastasis-mediating gene products is extensively regulated by posttranscriptional mechanisms. Collagenases are typically secreted as precursors whose activation requires proteolytic cleavage; collagenase type IV becomes active after cleavage by stromelysin while prostromelysin and interstitial procollagenase are activated by plasmin. Ligands for homing receptors often contain multiple domains. The heparin-binding amino-terminus of thrombospondin stimulates chemotaxis while the carboxy-terminus mediates haptotaxis in an RGD-inhibitable fashion. Comparably, a prerequisite for the interaction of the N-terminal osteopontin domain with integrin receptors is phosphorylation of the cytokine while the C-terminal domain engages variant CD44 by protein-protein interaction. The gene for the homing receptor CD44 contains ten variant exons that can be spliced into the extracellular domain and determine its engagement of various ligands. Differential effects on binding to extracellular matrix and hyaluronate also depend on the glycosylation and sulfation status of CD44. Posttranscriptional modification of function of these molecules may be beneficial in two ways. Activation by mechanisms such as proteolytic cleavage and phosphorylation can be accomplished quickly in stress situations; some of the precursor molecules are widely expressed and can acutely be converted at a site of damage. Also, diversity in structure may encode organ specificity in homing and metastasis formation (a "postal code" of sorts). In clinical diagnosis, tumors that grow in a locally invasive manner but do not form distal metastases, including cases of basalioma, glioblastoma, chondrosarcoma, and myelomonocytic leukemia, are often referred to as semimalignant. The molecular mechanisms of local invasion, however, are distinct from conventional forms of cancer only insofar as their target tissues are identical to the tissues of origin.

In conclusion, the topology of cancer spread is regulated by a set of developmentally nonessential genes that physiologically mediate inflammation, wound healing, and neovascularization. The function of their products is extensively regulated posttranscriptionally. The entity of these genes encodes the repertoire of stress responses which are predominantly executed by macrophages and lymphocytes. Metastasis-associated gene products therefore constitute a unique and essential group of cancer related biomolecules whose functions are distinct from those of growth control or senescence genes.

Regulation and dysregulation of metastasis genes

As in the case of yin and yang, phenomena in biology typically have a counterbalance. This also holds true for the regulation of cell dissemination. While tumor suppressor genes inhibit cell cycle progression and serve as antagonists for oncogenes, the genes that mediate metastatic spread are balanced by metastasis suppressor genes. The derived gene products typically are adhesion molecules that procure cell anchorage and inhibit migration. Expression of L-CAM is inversely correlated with the metastatic potential of various tumor cell lines. Loss of cadherin expression in squamous cell carcinomas of the head and neck, prostate cancer, and cancers of the female reproductive tract is associated with poor differentiation and high invasiveness. E-cadherin can prevent the invasive phenotype in T-lymphoma cells. Proteinases also have their antagonists. Tissue inhibitors of metalloproteinases negatively regulate invasion. Their overexpression reduces metastatic potential whereas antisense RNA enhances the malignant phenotype.

It could be argued that metastasis-associated genes are not, in strict terms, cancer genes because mutations in them have not been linked to the risk of contracting cancer. While it is true that these genes have not yet been observed to be mutated in malignancies as in the case of the classical oncogenes (frequently through point mutations, deletions, frame shifts, or translocations), they are subject to dysregulation. A case in point is the expression of ICAM-1 on melanoma cells, which is an indica-

tor of poor prognosis. Similarly, the homing receptor CD44 is often expressed on cancer cells but not at all on their benign precursors. Alternatively, cancer cells may display splice variants of this receptor which are not detected on their nontransformed counterparts. Therefore aberration of genes for cancer spread occurs frequently at the level of transcription or splicing. Without this dysregulation of gene expression tumors could not become malignant.

Metastasis genes and classical cancer genes: the big picture

Even though uncontrolled growth does not inevitably lead to metastatic spread, consistent patterns of organ preference by cancers of particular tissue origin suggest that there is a necessary connection between mutations of oncogenes or tumor suppressor genes and the expression of genes that mediate tumor dissemination. The molecular basis for this connection is currently largely unknown. Expression of metastasis-specific splice variants of CD44 and the oncogene *ras* are connected in an autocatalytic mode in which *ras* induces promoter activity for CD44 through an AP-1 binding site while transfection of CD44v enhances the expression of *ras*. This mutual induction may contribute to the perpetuation of cell division and spreading which are characteristic of malignancy. Motility-associated cytokines, including type IV collagenases and osteopontin, can also be induced by *ras* and similar relationships may apply for other oncogenes, including v-*mos*, v-*raf*, v-*fes*, and v-*src* [2].

Recent research has identified the genes that underlie the three phenotypic characteristics of cancer and has allowed a distinction between malignant and benign tumors at the molecular level. Only tumors in which the dysregulation of growth is associated with expression of genes whose products mediate dissemination become malignant. This attributes a central role in carcinogenesis to metastasis genes and metastasis suppressor genes. The definition of molecules that are rarely expressed in the healthy adult organism has given rise to the potential emergence of new drug targets. Among them are telomerase, structurally altered oncogene products such as fusion proteins or mutants, and also some of the stress response molecules that mediate metastasis formation. Prominently, blocking the integrin $\alpha_v\beta_3$, which is essential for tumor angiogenesis, has been successful in several experimental systems [3, 4]. Likewise, splice variants of CD44 that mediate dissemination of multiple cancers and are physiologically expressed on immune cells only after antigenic challenge have been targeted in experimental therapy with promising results [5, 6, 7]. Such progress provides the opportunity for a more successful broad attack on the cancer epidemic. As the profile of the killer becomes more refined the prospect for its containment improves.

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